IDEAS®

Image Data Exploration and Analysis Software
User's Manual

Version 6.0, March 2013

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Table of Contents

Patents and Trademarks ..... **ii**

End User License Agreement ..... **iii**

Disclaimers vi

Technical Assistance vi

Table of Contents ..... **vii**

Chapter 1: Introduction **1**

Welcome to IDEAS® 6.0 ..... **2**

How to use this manual 2

What's New in IDEAS 6.0 ..... **3**

Setting Up the IDEAS® Application **4**

Hardware and Software Requirements 4

Installing the IDEAS Application 4

Setting Your Computer to Run the IDEAS® Application 5

Viewing and Changing the Application Defaults 5

Chapter 2: Overview of IDEAS **13**

Comparing the FlowSight basic, Quantitative Imaging and ImageStream data files **15**

Understanding the Data Analysis Workflow **16**

Overview of the Data File Types **18**

Chapter 3: Getting Started with IDEAS **21**

Guided Analysis **23**

Application Wizards 24

Open File Wizard: 26

Display Properties Wizard 27

Begin Analysis Wizard 28

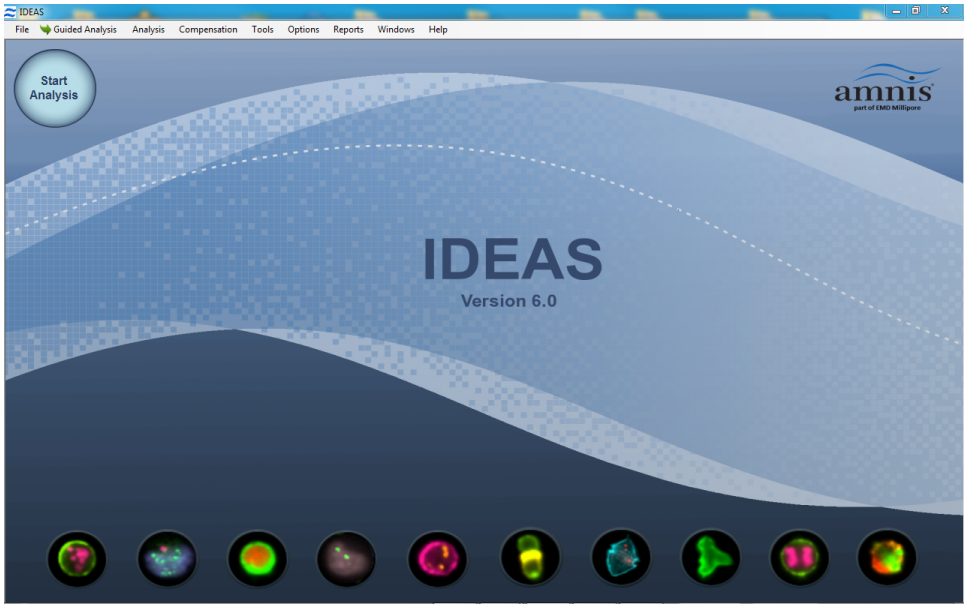
Feature Finder Wizard 29

Apoptosis Wizard 33
Cell Cycle - Mitosis Wizard 35
Co-localization Wizard 37
Internalization Wizard 39
Nuclear Localization Wizard 41
Shape Change Wizard 43
Spot Wizard 45
Building Blocks **47**
Advanced Analysis **49**
Opening data files **50**
Saving Data Files **56**
Overview of Compensation **58**
Merging Data Files **69**
Creating New Data Files **71**
Viewing Sample Information **72**
Batch Processing **74**
Overview of the Data Analysis Tools **78**
Overview of the Image GalleryUsing the Image Gallery **79**
Overview of the Image Gallery 79
Setting the Image Gallery Properties 81
Working with Individual Images 86
Overview of the Mask Manager **88**
Overview of the Mask Manager 88
Creating New Masks with the Mask Manager 88
Viewing and Editing a Mask 91
Example of Creating a Mask 92
Overview of the Analysis Area **94**

Analysis Area Tools 94
Creating Graphs 95
Creating Regions on Graphs 99
Analyzing Individual Images 103
Viewing the Object Feature Values 108
Adding Text to the Analysis Area 110
Population Statistics 111
Using the Feature Manager 113
Overview of the Feature Manager 113
Viewing feature definitions 114
Creating New Features with the Feature Manager 114
Ranking features by discriminating power 118
Using the Population Manager 124
Creating Tagged Populations 127
Using the Region Manager 129
Creating Reports and Exporting Data 131
Viewing and Changing the Application Defaults 132
Setting the Image Gallery Properties 138
Reporting Images and Graphs 144
Prepare the image gallery and analysis area for reporting. 144
Copy full or partial screens 144
Print the analysis area or image gallery directly 145
Copy images to the clipboard or save images to files 145
Copy graphs to the clipboard or save graphs to a file 146
Reporting Statistics 148
Define a Statistics Report 148
Generating a Statistics Report using .daf Files 150

Reporting Statistics from a Single Graph or Statistics Table 151
Exporting Data **152**
Creating TIFs From Population for Export **154**
Printing Data **155**
Chapter 4: Features and Masks **157**
Understanding the IDEAS® Features and Masks **158**
About Features 159
Features Categories 161
Table of Base Features - Alphabetical **162**
Table of Base Features- by Category **164**
Table of Basic Features available for FlowSight without QI **169**
Understanding the Size Features **170**
Understanding the Location Features **182**
Understanding the Shape Features **195**
Understanding the Texture Features **205**
Understanding the Signal Strength Features **216**
Understanding the Comparison Features **234**
Understanding the System Features **242**
Camera Timer Feature **244**
About Masks **250**
Appendix: Troubleshooting **269**
Glossary **273**
Index **275**

Chapter 1: Introduction



Welcome to IDEAS® 6.0

Welcome to the IDEAS version 6 application documentation for ImageStream and FlowSight data analysis.

How to use this manual

This manual provides instruction for using the Amnis IDEAS® application to analyze data files from the Amnis ImageStream and FlowSight cell analysis systems.

The intuitive user interface of the IDEAS application makes it easy for you to explore and analyze data. The application can quantify cellular activity by performing statistical analyses on thousands of events and, at the same time, permit visual confirmation of any individual event. Furthermore, you can operate the application in a batch processing mode and store specific analysis templates for either repeated use or sharing with colleagues.

The fastest way to put the IDEAS application to work is to first skim through this manual—becoming familiar with the application's structure, compensation, file types, and analysis tools—and then use the application wizards on some sample experimental data to begin exploring the power that the application provides. This manual has been integrated into the IDEAS application to provide searchable and context sensitive help. Typing F1 while in the application opens the help files.

What's New in IDEAS 6.0

IDEAS 6.0 offers numerous improvements for analyzing data from any ImageStream or FlowSight instrument. Please refer to the web site for the latest improvements and updates to this manual.

1 General
   - New user application default settings include setting graph and statistics exporting formats.
   - Color mapping tool has been improved in the application defaults settings.
   - Files processed prior to IDEAS version 4.0 may not load in 6.0.

2 Data
   - New Feature Finder wizard for choosing a feature.
   - Tagging tool updates the number tagged.
   - Tagging tool defaults the population symbol to a solid diamond and a new color for each population for easier viewing in graphs.

3 Features and Masks
   - The Valley mask has been improved.

4 Image Gallery
   - The measurement tool is now available in the gallery.

5 Analysis Area
   - Moving panels in the analysis area is now easier. Click and drag on the panel anywhere except the toolbar.
   - Panels are aligned by their tops by lines.
   - Cursor changed to indicated current state.

6 Reporting
   - Copy/Save graphs and images has been improved. Images may show scale bar, adjust font sizes, and save as .tif, .png, or .bmp files at 300 DPI. Graph default settings may be used for fonts, graph sizes, and resolution.

Setting Up the IDEAS® Application

This chapter describes the hardware and software requirements for the application, which includes the procedures for installing, removing, and upgrading the application. The following subsections cover this information:

Setting Up the IDEAS® Application

Installing the IDEAS Application

Setting Your Computer to Run the IDEAS® Application

Viewing and Changing the Application Defaults

Hardware and Software Requirements

This section states the minimum and the recommended hardware and software requirements for running the IDEAS application.

Hardware Requirements

The minimum hardware requirements are 4 GB of RAM and a dual core Intel processor. However, due to the large size of the image files that the ImageStream cell analysis system creates, a larger amount of RAM will prevent paging and thus increase performance.

Software Requirements

IDEAS 6.0 64 bit version requires that the Windows 7 operating system be installed on your computer. IDEAS 32 bit version requires Windows XP, Windows 2000 or later version of the operating system. The latest service pack and any critical updates for the operating system must be included. You must also install the Microsoft .NET Framework 2.0, which requires Microsoft Internet Explorer 5.01 or later.

Important: If the software requirements are not met, Setup will not block installation nor provide any warnings.

Note that service packs and critical updates are available from the Microsoft Security Web Site.

Installing the IDEAS Application

If the IDEAS application has previously been installed, the previous version will be removed during installation.

To install IDEAS software

1 Download the application Setup file from your account at www.amnis.com.
2 View the contents in My Computer or Windows Explorer.
3 Double-click `Setup.exe`.
4 Follow the instructions until the installation process has completed.

5 MadCap help viewer is installed and opened during installation or upgrade.
6 Select the language and check box to not show this dialog again.
7 An icon appears on the desktop and IDEAS Application appears on the All Programs menu.

Setting Your Computer to Run the IDEAS® Application

Setting the Screen Resolution

Confirm that the screen resolution is adequate for the IDEAS application. To be able to view the entire application window, you must set the width of the screen resolution to a minimum of 1024 pixels.

To set the screen resolution

1 From the Start menu, select **Control Panel**, and then click **Display**.
2 Click the **Settings** tab to set the screen resolution.

Viewing File Name Extensions

When loading a file, the IDEAS application uses the file name extension to determine the file type. It will be easier for you to distinguish raw image files, compensated image files, and data analysis files from each other if Windows Explorer does not hide the extensions.

To view file name extensions

1 In Windows Explorer, go to **Tools** > **Folder Options**.
2 Click the **View** tab, and make sure that the Hide extensions for known file types check box is not selected.
3 Click **OK**.

Copying the Example Data Files

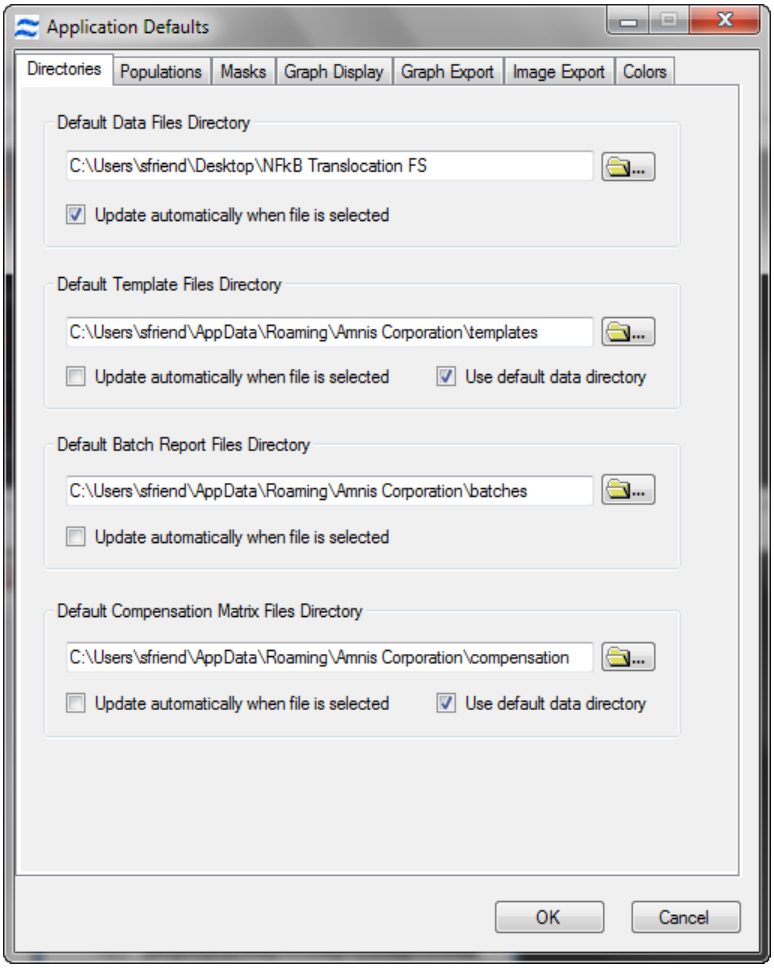
Copy data files to a single directory on your hard drive. Sample data files are available on your workstation or at www.amnis.com/login for customers with an account.

To copy the example data files

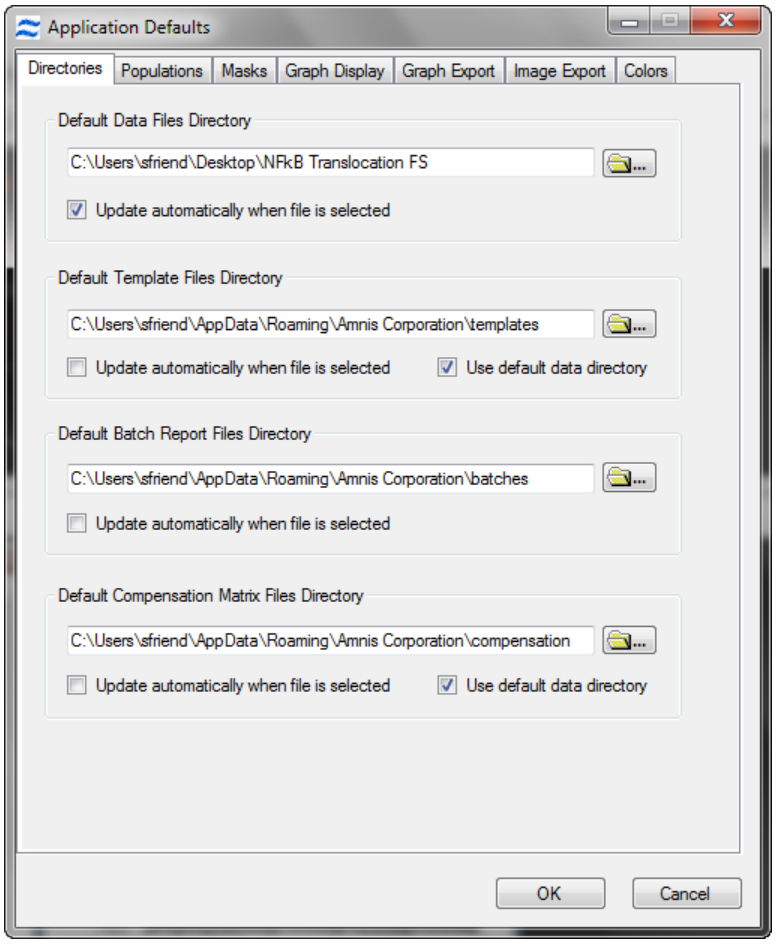
1 Copy the data files.
2 Right-click the directory that contains the data files, and click **Properties**.
3 Clear the **Read-only** check box.
4 Click **OK**.

Viewing and Changing the Application Defaults

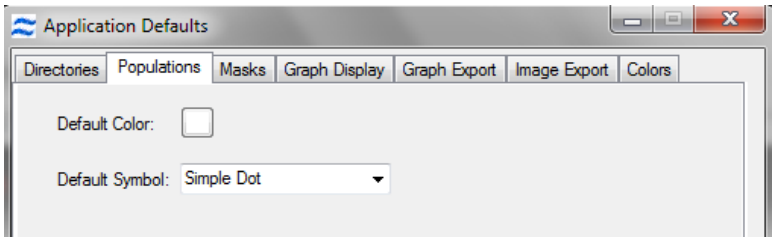
To view or change these defaults, choose**Application Defaults** from the **Options** menu, Each tab allows you to view or change the default settings.



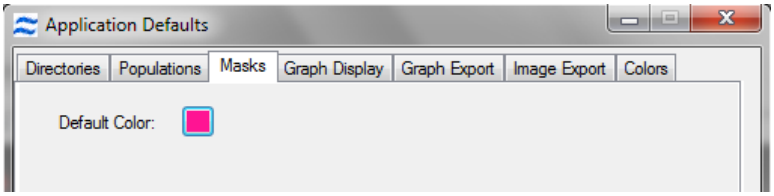
The **Directories** tab contains the default Data, Template, Batch or Compensation Matrix file directories.



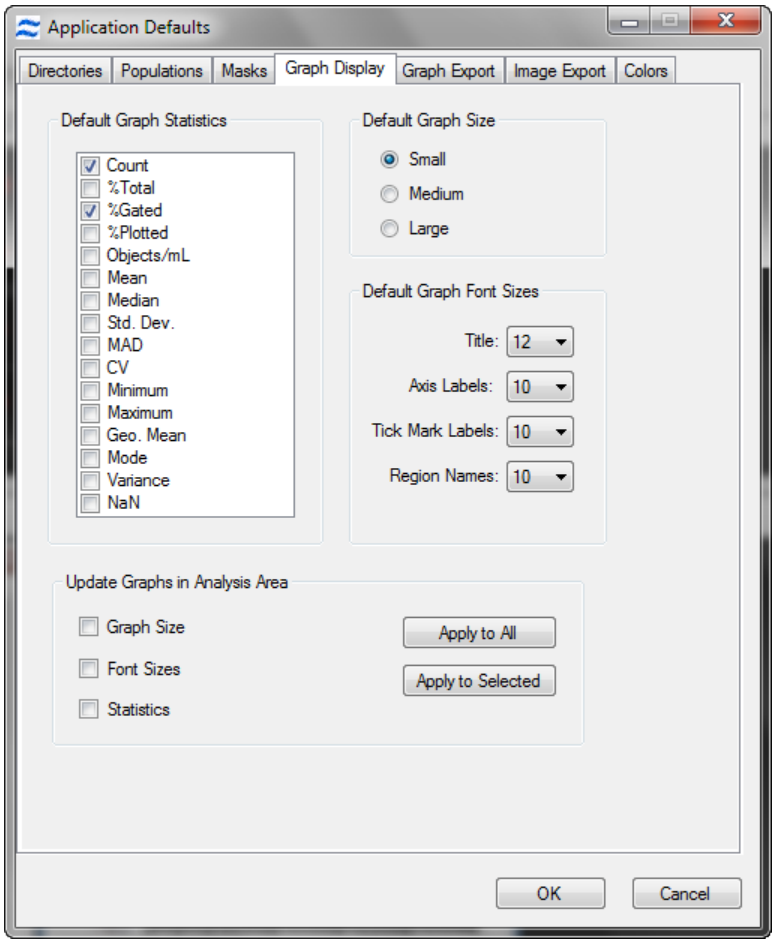
The **Populations** tab contains the default color or symbol for populations. To change the default settings, click on the color to or choose a default symbol from the list.



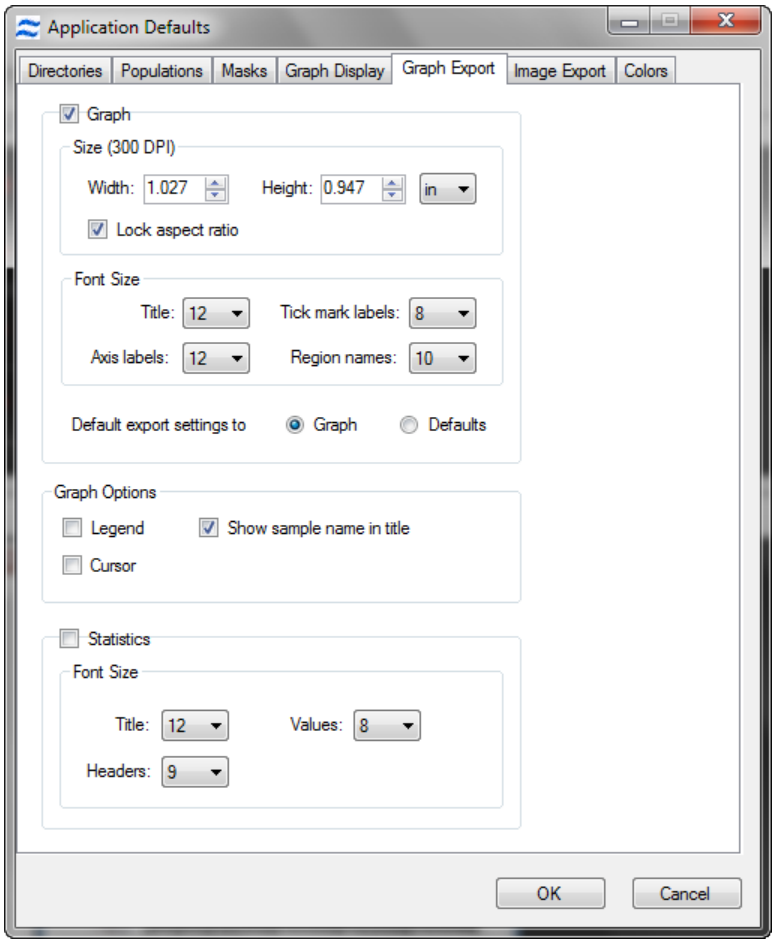
The **Masks** tab contains the default mask color. To change the color of the mask click on the color button.



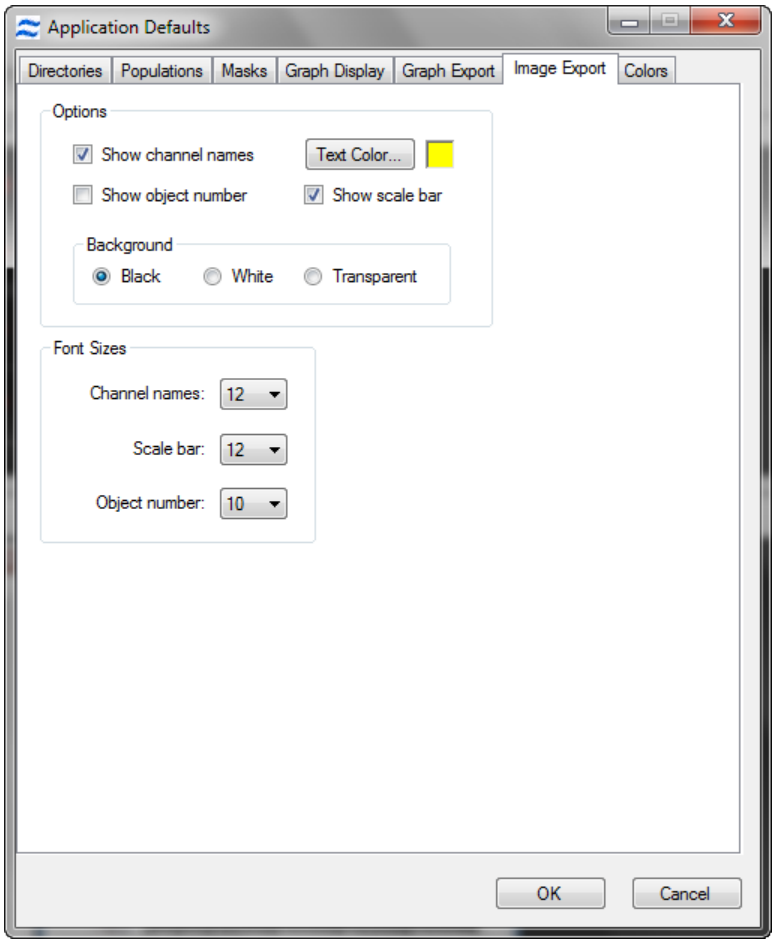
The **Graph Display**tab contains the default list of statistics shown for a graph. Check the box next to the statistic to have it show below the graphs when statistics are shown for a graph. The default graph size and font size for the graphs in the analysis area may be changed in this tab.



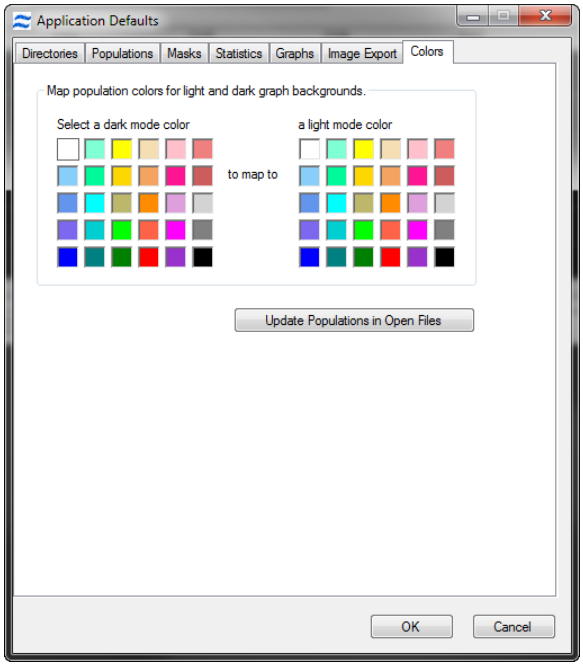
The **Graph Export** tab contains the default settings for printing and exporting graphs when copying and pasting from IDEAS for reporting into other programs.



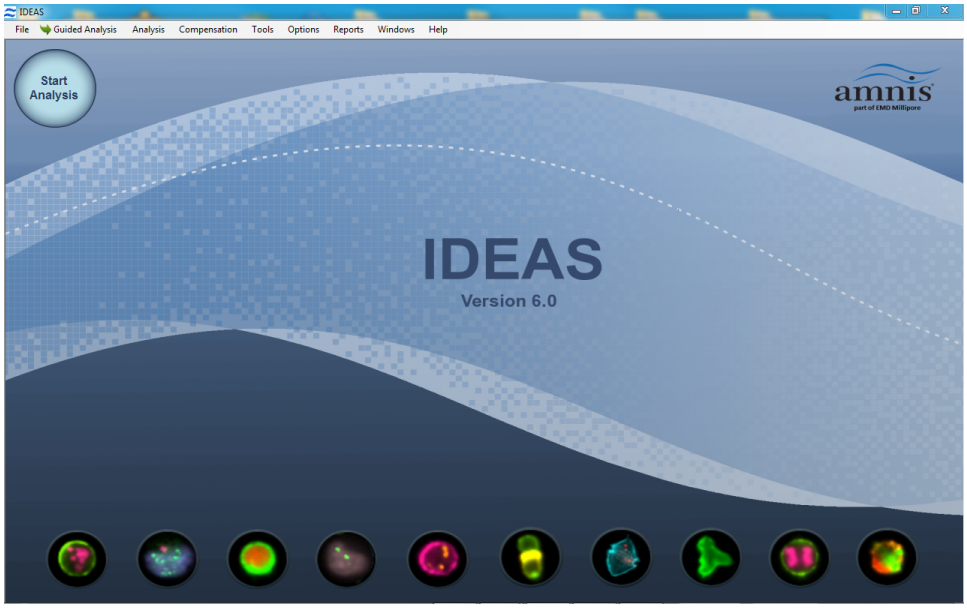
The **Image Export** tab contains the default settings for image export when copying and pasting from IDEAS for reporting into other programs.



The **Colors** tab contains the mapping of dark and light mode colors.



Chapter 2: Overview of IDEAS



Overview of the IDEAS® Application

This chapter provides an overview of the IDEAS application user interface, the files that the IDEAS application creates and uses, the recommended directory organization and an overview of the work-flow.

Understanding the Data Analysis Workflow

Overview of compensation, analysis tools and file structure

The Amnis cell analysis systems possess unique capabilities that neither flow cytometry nor microscopy alone can deliver. The IDEAS application provides an image gallery to view every cell in the data file along with linked graphical data for confident gating and image confirmation. The application contains powerful algo-rithms that facilitate and quantify the image analysis of ImageStream and FlowSight QI data. Examples include the analysis of molecule co-localization and trans-location, the analysis of cell-to-cell contact regions and signaling interactions, the detection of rare molecules and cells, and a comprehensive classification of large numbers of cells. The IDEAS application acquires data from INSPIRE™, com-pensates the images, and allows the user to evaluate images with data analysis tools. For more information about the QI and non-QI FlowSight data see Comparing the FlowSight basic, Quantitative Imaging and ImageStream data files.

Comparing the FlowSight basic, Quantitative Imaging and ImageStream data files

There are three types of instruments that collect data for Image Analysis in IDEAS: The FlowSight without Quantitative Imaging (QI), The FlowSight with the QI upgrade and the ImageStream. There are some differences in the available features and analysis that can be done. The table below outlines these differences.

Data comparison

	FlowSight - basic	FlowSight - QI	ImageStream
Default template	INSPIRE features and acquisition analysis	IDEAS default feature set - analysis	IDEAS default feature set - no analysis
Default compensation matrix	INSPIRE matrix	No compensation	No compensation
New feature calculation	Combined features only	Unlimited	Unlimited
User defined masks	No	Yes	Yes
Default mask	INSPIRE mask calculated during acquisition	Default (Object) mask computed in IDEAS	Default (Object) mask computed in IDEAS
Wizards	Open File, Display Properties and Begin Analysis	Complete set	Complete set
Building blocks	All	All	All
Merging files	No	Yes	Yes
Compensation	Intensity and Images only	All features and Images	All features and Images
Create new files from populations	No	Yes	Yes
IDEAS	Requires version 5.0.x or later	Requires version 5.0.x or later	Can open in 4.0 or later

.

Understanding the Data Analysis Workflow

Data analysis in IDEAS begins with opening a raw image file (.rif) that was collected and saved using INSPIRE. Then, an existing compensation matrix or a new compensation matrix is applied to the .rif file and two additional files are created, the .cif (compensated image file) and .daf (data analysis file).

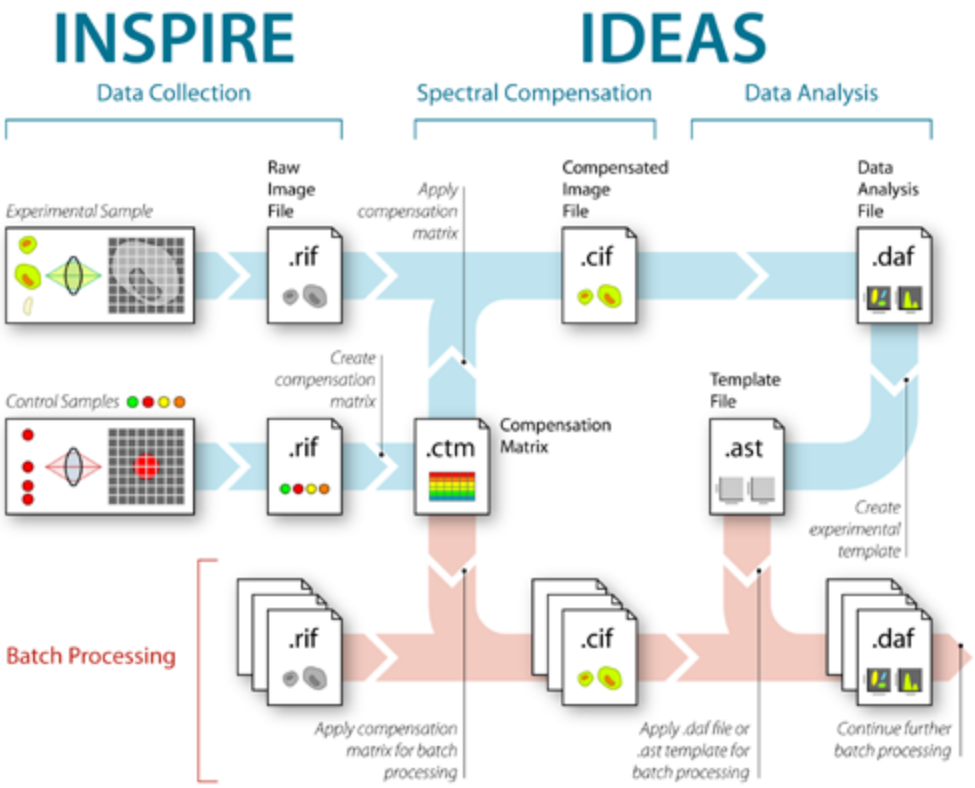
A compensation matrix performs fluorescence compensation, which removes fluorescence that leaks into other channels. See Overview of Compensation for more information about compensation. A compensated image can accurately depict the correct amount of fluorescence in each cell image. Compensation is defined as the correction of the fluorescence crosstalk. When creating the .cif the IDEAS application also automatically performs corrections to the raw imagery using values saved from the instrument at the time of data collection. These corrections include flowspeed normalization, brightfield gains, and spatial registry.

A template is used to define the features, graphs, image display properties and analysis for the .daf. Within the .daf file, the user can perform many analyses using the tools and wizards within the application and save the results as a template file (.ast).

The IDEAS application then calculates feature values and shows the data as specified by the selected template.

Once a data analysis file (.daf file) or compensated image file (.cif file) is saved, it can be opened directly for data analysis. You would only open a .cif if you wanted to change the template or a .rif file to change the compensation.

The diagram on the next page displays this workflow.



Overview of Data Analysis Workflow

1 Create a compensation matrix using the single color control files. Open an experimental .rif file or from the Compensation menu choose Create New Matrix.

2 A .cif and .daf file are automatically created. Analyze the experimental file using data analysis tools in the .daf file to create an analysis template.

3 Create a statistics report table within the .daf file and save the data file, as an anlaysis template.

Note: this is usually done on the positive and negative controls to create the appropriate analysis and then applied to the rest of the experimental files in the next step.

4 Perform batch processing, applying compensation and template files created above.

Overview of the Data File Types

Data from the Amnis cell analysis systems are collected and managed using three types of data files: raw image file (.rif), compensated image file (.cif), and data analysis file (.daf).

This section describes each file type and the table summarizes the features of each file.

Raw Image File (.rif)

The INSPIRE application saves the digital image data, pixel intensities and location that were acquired by the instrument to a .rif file.

A .rif file contains:

- Pixel intensity data (counts and location) collected for each object that the instrument detected.
- Instrument settings that were used for data collection.
- Calibration values from ASSIST.
- Compensation matri x if used while acquiring data.

Compensated Image File (.cif)

The IDEAS application creates a .cif file when the user opens a .rif file and applies a compensation matrix. The segmentation algorithm automatically defines the boundaries of each object, creating a mask that is used for calculating feature values. The applied compensation matrix performs pixel-by-pixel fluorescence compensation prior to segmentation.During the creation of the .cif file, the application makes corrections to the imagery. These corrections include:

- Removal of artifacts from variability in the flow speed, camera background, and brightfield gains.
- Alignment of the objects to subpixel accuracy, which allows the viewing of compensated imagery, composite imagery and the calculation of multi-image feature values, such as the Internalization value.
- Coincident objects are cut apart to place into individual image frames. Note that this will increase the number of objects in the file.

Multiple .cif files can be created from a single .rif file by applying a different fluorescence compensation matrix or correction each time a .rif file is opened and choosing a unique name for the .cif file. Similarly, you can create a new .daf file from a single .cif file by creating a new name and applying a different analysis template.

Data Analysis File (.daf)

The IDEAS application creates a .daf file while it is loading a .cif file into a template file (.ast). The .daf file is the interface to visualize and analyze the imagery that the .cif file contains and must reside in the same directory as the corresponding .cif file.

The .daf file contains:

- Feature definitions
- Population definitions
- Calculated feature values
- Image display settings
- Definitions for graphs and statistics

Loading a .daf file restores the application to the same state it was in when the file was saved, i.e., with the same views, graphs, and populations. In IDEAS versions 3.0 or later, a .daf file may be used as a template.

Note: When a .daf file is opened, the .cif file must be located in the same directory as the .daf file since the .daf file points to images used for analysis that are stored in the associated .cif file.

Template (.ast)

The IDEAS application saves the set of instructions for an analysis session in a .daf file to a template (.ast file). Note that a template contains no data; it simply contains the structure for the analysis. This structure includes definitions for:

- Features
- Graphs
- Regions
- Populations

The .ast also contains settings for:

- Image viewing
- Image names
- Statistics

The `\templates` subdirectory (under the directory where the IDEAS application was installed) contains the default template, named defaulttemplate.ast. Because a template is required for loading a .cif file, you must use the default template to create the first .daf file. After you save a custom template, you can use it for any subsequent loads of .cif files.

Note: The default template may change between releases of the IDEAS application software. In IDEAS versions 3.0 or later, a .daf file may be used as a template. The default template contains over 200 calculated features per object. An expanded template is also available that includes over 600 calculated features per object. The FlowSight without the Quantitative Imaging upgrade has a limited set of features available.

Compensation Matrix File (.ctm)

The IDEAS application saves the compensation values that are created and saved during the spectral compensation of control files to a compensation matrix file (.ctm file). This file has no associated object data; it is created and saved to be applied to experimental files. The compensation matrix can be created in IDEAS using single

color control files after acquisition or during acquisition. See the INSPIRE or FlowSight manual for more information.

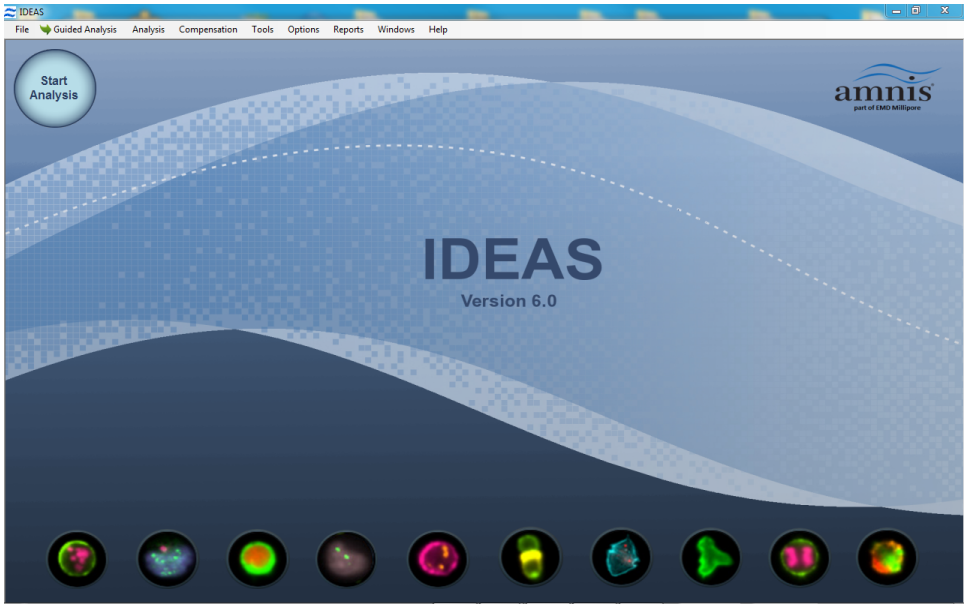
Review of Data File Types

Table 1: Review of Data File Types

File Acronym and Name	File Creation	Description
.rif Raw Image File	Created in INSPIRE	Contains instrument setup data, pixel intensity data, and uncorrected image data from the INSPIRE application. The IDEAS application uses the .rif file to create a compensated image file (.cif file).
.cif Compensated Image File	User creates a .cif from the .rif and .ctm	Contains imagery that has been corrected for variations in the camera background, camera gains, flow speed, and vertical and horizontal positioning between channels. Serves as a database of images used for feature-value calculations and imagery display. The IDEAS application also performs fluorescence compensation, if necessary, when creating a .cif file. The IDEAS application loads the .cif file into a template to create a data analysis file (.daf file)
.daf Data Analysis File	References the .cif	The main working data file that contains the calculated feature values, the graphs, and the statistics used for analysis. The .daf file references the .cif.
.ast Template File	Created from the .daf	This file contains no data; it contains the structure for the analysis, such as, definitions for features, graphs, regions, and populations; image viewing settings; image names; and statistics settings.
.ctm Compensation Matrix File	User creates new .ctm when opening a .rif or during acquisition	Contains compensation values that are created and saved during the spectral compensation of control .rif files. This file has no associated object data; it is created and saved to be applied to experimental .rif files.

Note about Case Sensitivity: Even though Windows does not treat file names as case sensitive, the IDEAS application depends on the case-sensitive .rif, .cif, and .daf file name extensions to identify the file types. Avoid the use of illegal characters for file names such as: “\/:*?<>!”.

Chapter 3: Getting Started with IDEAS



Getting Started with the IDEAS Application

This chapter is divided into two sections. First, guided analysis is described using the analysis wizards and second, advanced analysis with more detailed instructions that describe how to open, compensate, merge, save, and create data files without using the wizards. Building blocks are also discussed which provide a short cut method to building commonly used graphs.

Guided analysis makes it easy to start analyzing your data. Once you are familiar with the basic analysis available you may want to perform more advanced analysis.

Note that data files from FlowSight without the Quantitative Imaging upgrade have a limited feature set and limited wizard analysis.

General Outline of data analysis:

Note that these steps apply to any type of data analysis whether you use a wizard or not.

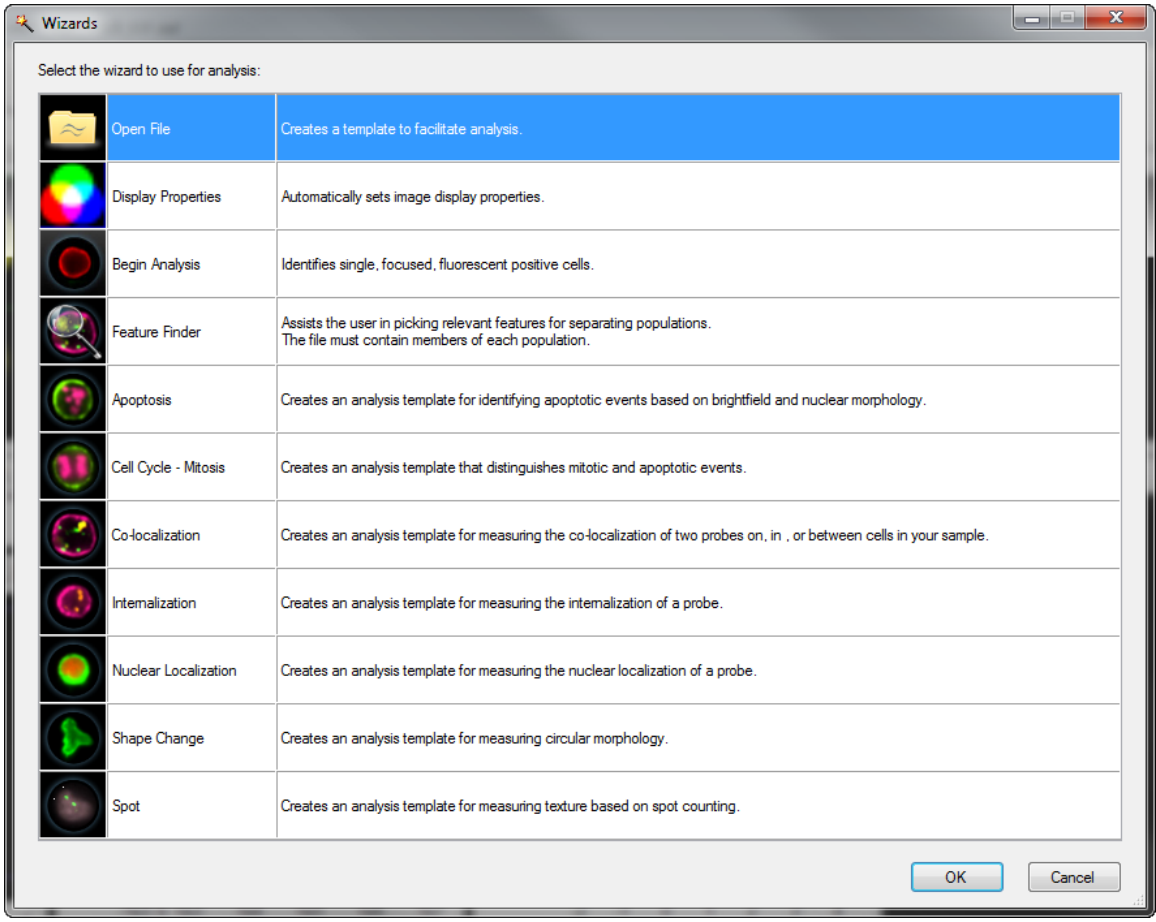
1. Open one data file (the + or - control).
2. Create and save a compensation matrix for the experiment.
3. Using an application wizard or the begin analysis wizard:
   - Select focused cells.
   - Select single cells (or conjugates).
   - Select channels for subpopulation markers and gate to define subpopulations.
   - Gate on positive cells for the channels you wish to use for morphological analysis.
4. By using an application wizard evaluate the feature for your analysis and refine as needed. Or follow the feature finder wizard to find the feature that separates your populations.Note that if the morphological differences are in separate files this may require merging both a + and - control before beginning step 1.
5. Refine the analysis and save the template.
6. Perform batch analysis on all data files in the experiment using the compensation matrix and analysis template.

Guided Analysis

Data analysis always begins with opening a data file. The Start Analysis button will step you through opening a file, setting the image display mapping and choosing an analysis wizard.

Application wizards are available to guide you through an analysis. The wizards can be accessed from the Guided Analysis menu or the wizard tool or at the end of the Start Analysis routine.

The following wizards are available:


Wizards
Select the wizard to use for analysis:
Open File
Creates a template to facilitate analysis.
Display Properties
Automatically sets image display properties.
Begin Analysis
Identifies single, focused, fluorescent positive cells.
Feature Finder
Assists the user in picking relevant features for separating populations.
The file must contain members of each population.
Apoptosis
Creates an analysis template for identifying apoptotic events based on brightfield and nuclear morphology.
Cell Cycle - Mitosis
Creates an analysis template that distinguishes mitotic and apoptotic events.
Co-localization
Creates an analysis template for measuring the co-localization of two probes on, in , or between cells in your sample.
Internalization
Creates an analysis template for measuring the internalization of a probe.
Nuclear Localization
Creates an analysis template for measuring the nuclear localization of a probe.
Shape Change
Creates an analysis template for measuring circular morphology.
Spot
Creates an analysis template for measuring texture based on spot counting.
OK
Cancel

Application Wizards

General:

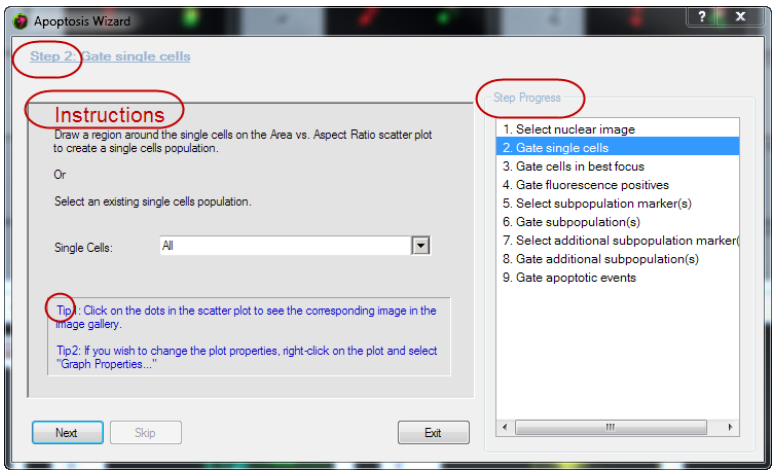
- Open File Wizard:
  - Guides you through the process of opening a data file and setting image display mapping in the image gallery.
- Display Properties:
  - Sets image display mapping in the image gallery.
- Begin Analysis:
  - Guides you through finding focused, single, positive cells.
- Feature Finder:
  - Guides you through finding focused, single, positive cells.

Application specific:

- Apoptosis Wizard
  - Guides you through the process of creating the features and graphs for analyzing apoptosis.
- Cell Cycle - Mitosis Wizard
  - Guides you through the process of creating the features and graphs for analyzing the cell cycle and enumerating mitotic events.
- Co-localization Wizard
  - Guides you through the process of creating the features and graphs for analyzing the co-localization of 2 probes.
- Internalization Wizard
  - Guides you through the process of creating the features and graphs for analyzing the internalization of a probe.
- Nuclear Localization Wizard
  - Guides you through the process of creating the features and graphs for analyzing the nuclear localization of a probe.
- Shape Change Wizard
  - Guides you through the process of creating the features and graphs for analyzing the circular shape of a cell using a surface stain or brightfield image.
- Spot Wizard
  - Guides you through the process of creating the mask, feature and graphs for analyzing fluorescently labeled spots in images.

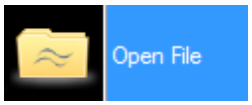
The wizard window is organized so that the instructions for each step are written in the left side of the window, the stepwise progress through the wizard is shown in the list on the right side and there may be tips provided at the bottom of the window.

Click Next to progress through the wizard or Exit to stop at any time. Some steps are optional and a Skip button is provided. Follow the instructions in the wizard to complete an analysis.


Apoptosis Wizard
Step 2: Gate single cells
Instructions
Draw a region around the single cells on the Area vs. Aspect Ratio scatter plot to create a single cells population.
Or
Select an existing single cells population.
Single Cells:
All
Tip1: Click on the dots in the scatter plot to see the corresponding image in the image gallery.
Tip2: If you wish to change the plot properties, right-click on the plot and select "Graph Properties..."
Next
Skip
Exit
Step Progress
1. Select nuclear image
2. Gate single cells
3. Gate cells in best focus
4. Gate fluorescence positives
5. Select subpopulation marker(s)
6. Gate subpopulation(s)
7. Select additional subpopulation marker(
8. Gate additional subpopulation(s)
9. Gate apoptotic events

Open File Wizard:

This wizard will guide you through the opening of a data file and setting the image display mapping. Use this wizard to open a file if you are not using one of the application specific wizards.

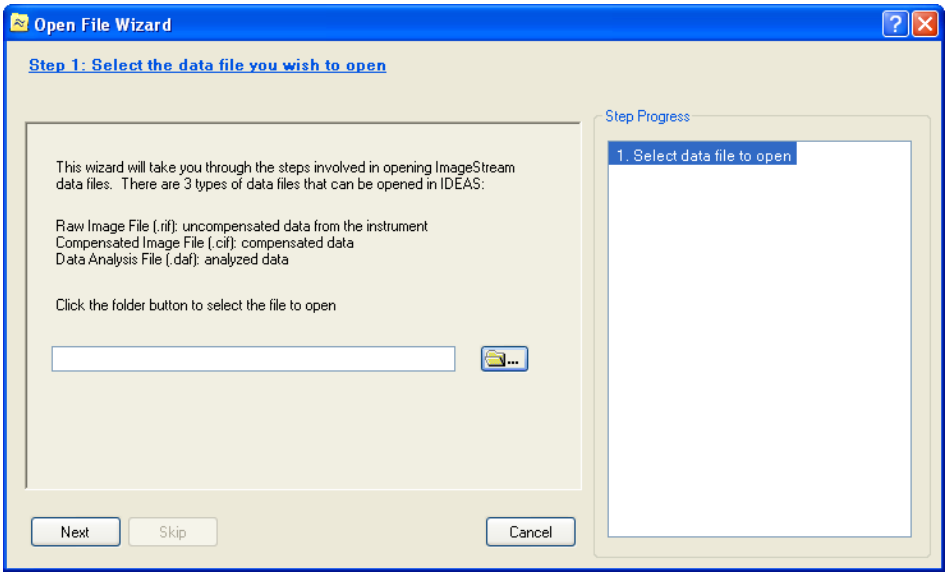


To begin, double-click on Open File

Follow the instructions to open your file.

Tip: You can limit the view to specific file types (.daf, .cif or .rif) by using the drop-down menu 'Files of type:' in the Select Data File window.

A .daf file will open directly without further input, a .cif file will require a template and a .rif file will require a template and a compensation matrix. If the template or compensation matrix boxes are left blank, the default template and/or matrix will be applied. For more information on opening data files see Opening data files .


Open File Wizard
Step 1: Select the data file you wish to open
This wizard will take you through the steps involved in opening ImageStream data files. There are 3 types of data files that can be opened in IDEAS:
Raw Image File (.rif): uncompensated data from the instrument
Compensated Image File (.cif): compensated data
Data Analysis File (.daf): analyzed data
Click the folder button to select the file to open
Step Progress
1. Select data file to open
Next
Skip
Cancel

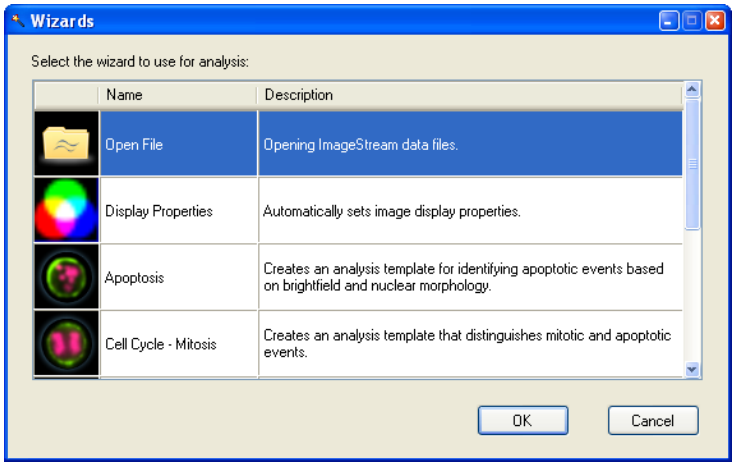
Once a data file is open you may begin analysis.

Display Properties Wizard

This wizard is automatically run when you use the other analysis wizards or the Open File wizard. It is also available to run in any open data file from the Guided Analysis menu or from the wizard icon. This wizard will set the image display mapping for the channel images you select and create a view of selected images. Brightfield and scatter images will be automatically selected.

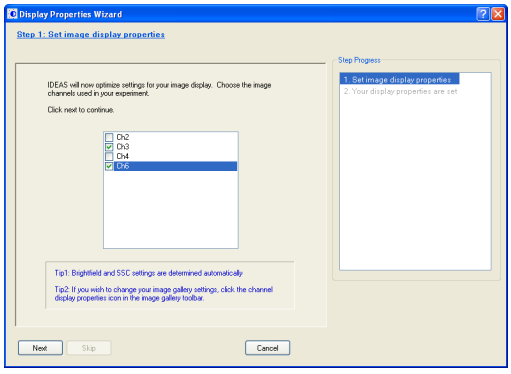
To begin, select wizards from the Guided Analyis menu or click the wizard icon in the analysis area toolbar.

The Wizards window opens.



Double-click on the Display Properties option and follow the instructions.

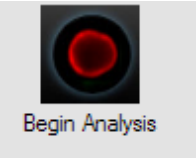
The Display Properties adjusts the mapping of the pixel intensities to the display range for optimizing the display and creates a view that includes the chosen channels. This is for display only and does not effect the pixel values. For more information on image display see Setting the Image Gallery Properties.



Begin Analysis Wizard

This wizard is available once a data file is open and will guide you through choosing the focused cells, then single cells, then choosing subsets of fluorescent positive cells for phenotypic analysis before progressing on to a morphological analysis.

Open a data file using the Start Analysis button or by choosing Wizards from the Guided Analysis menu. The wizards selection screen will appear once the data file is open. If you have an open data file and want to access this wizard, choose Wizards from the Guided Analysis menu.



To begin, double-click on Begin Analysis

Step 1. Gate cells in best focus

A histogram of the brightfield channel Gradient RMS values for the 'All' population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 2. Gate single cells

A scatter plot of the brightfield Area versus Aspect Ratio for the population chosen in step one has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.You may choose an already existing population.

Step 3. Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot to the analysis area. Click Skip if you do not wish to use this step. Draw regions in the scatter plot to identify as many populations as you want. This step will be repeated until you choose Skip or Finish.

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Feature Finder Wizard

The feature finder wizard will guide you through the process of choosing a feature for morphological analysis when a specific application wizard is not appropriate. This wizard is available once a data file is open and will guide you through choosing the focused cells, then single cells, then choosing subsets of fluorescent positive cells for phenotypic analysis before progressing on to choosing a feature for morphological analysis.

Open a data file that contains images of both phenotypes you wish to separate.Note that it may be necessary to merge two files together if the populations are in separate files. Open the data file using the Start Analysis button or by choosing Wizards from the Guided Analysis menu. The wizards selection screen will appear once the data file is open. If you have an open data file and want to access this wizard, choose Wizards from the Guided Analysis menu or click the wand icon.

Feature Finder

To begin, double-click on Feature Finder

Step 1. Gate cells in best focus

A histogram of the brightfield channel Gradient RMS values for the 'All' population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population. Click Next.

Step 2. Gate single cells

A scatter plot of the brightfield Area versus Aspect Ratio for the population chosen in step one has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.You may choose an already existing population.Click Next.

Step 3. Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity and include the channel(s) you are going to use for morphological analysis. Click Next to add the scatter plot to the analysis area. Click Skip if you do not wish to use this step. Draw regions in the scatter plot to identify as many populations as you want. This step will be repeated until you choose Skip or Finish.

Step 4. Assign truth populations

Choose two truth populations of cells that represent one morphological phenotype difference you wish to separate. Use the tagging tool icon to hand-tag images or select pre-existing gated populations. Note: If there are more than one phenotypic differences, repeat the process for each with new truth populations.

Step 5. Select channels and feature categories

Choose the channel and feature category you wish to explore. Multiple rows may be entered. The features in the table below will be created and calculated. All of the default features, newly created features and user defined features in the chosen categories will be evaluated for their ability to separate the truth populations. The three highest ranking features for each category will be saved and available for evaluation.

Step 7. Results

The top ranking features are listed in the table with their category and channel. A Statistics table is added to the analysis area that lists the features with the RD Mean for the truth populations. RD is the Fischer's discriminant ratio which is the difference in the means divided by the sum of the standard deviations for the two populations. The larger the RD value, the better the separation afforded by the feature. A scatter plot is added to the analysis area of the truth populations for the top two features.

It may be necessary to refine your results. Visually verify the morphology and separation for the features listed. Additional features may be quickly plotted by selecting them in the list and clicking 'Plot Features'. To return to the truth population assignment step click 'Refine populations'. To choose different channels or categories click 'Change Category'.

Table 1: List of additional features (beyond default) that are created and explored in the Feature Finder wizard

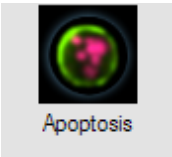
Feature category	Feature name and description	Mask used
Location	**Location Features are in X,Y pixel coordinates from an origin in the upper left corner, pixels or contour**	
	Delta Centroid XY Feature The distance between the Centroids of the intensity weighted and non-intensity weighted image	channel mask
Shape	**Shape Features define the mask shape and have units that vary with the feature.**	
	Aspect Ratio Feature The ratio of the Minor Axis divided by the Major Axis.	Object
	Circularity Feature The degree of the mask's deviation from a circle.	Object
	Compactness Feature Describes the density of intensities within the object.	Object
	Elongatedness Feature The ratio of the Height/Width which use the bounding box.	Object
	Lobe Count Feature	Object

Feature category	Feature name and description	Mask used
	The number of lobes in a cell. (Also see Symmetry)	
	Shape Ratio Feature The ratio of Thickness Min/Length features.	Object
	Symmetry 2, 3, 4 Features These three features measure the tendency of the object to have a single axis of elongation, a three-fold and a four-fold variation of the shapes.	Object
Size	**Size-based Features are in microns.**	
	Area Feature The size of the mask in square microns.	Object, Threshold 30,50,70 %
	Height Feature Based on a bounding rectangle, the Width is the smaller side and the Height is the longer side of the rectangle.	Object
	Length Feature Measures the longest part of the mask.	Object
	Major Axis and Minor Axis Features Describes the widest part of the mask and the narrowest part of the mask, respectively.	Object
	Perimeter Feature Describes circumference of the mask.	Object, Threshold 30,50,70 %
	Thickness Max Feature Describes the longest width of the mask.	Object
	Thickness Min Feature Describes the shortest width of the mask.	Object
	Width Feature Based on a bounding rectangle, the Width is the smaller side and the Height is the longer side of the rectangle.	Object
Texture	**Texture features measure pixel or regional variation and indicate the granularity or complexity of the image.**	
	Contrast Feature Enumerates changes of pixel values in the image to measure the focus quality of an image.	Morphology, Object
	H Texture Features Measures Haralick texture features. Granularity settings 1,5,15,19	Channel mask
	Modulation Feature Measures the intensity range of an image, normalized between 0 and 1.	Morphology, Object
	Spot Count Feature Enumerates the number of spots.	Channel mask
	Std Dev Feature Describes the overall distribution of pixel intensities.	Morphology, Object
Signal Strength	**Signal Strength Features are measured in pixel values.**	
	Intensity Feature The sum of the pixel intensities in the mask, background subtracted.	Morphology, Object, Threshold 30,50,70 %

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Apoptosis Wizard

The apoptosis wizard will guide you through the process of creating the features and graphs to measure apoptosis using the images of the nuclear dye and brightfield. Begin by opening a data file and then choosing the Apoptosis wizard.



To begin, double-click on Apoptosis

Follow the instructions to analyze your file.

Step 1. Select the nuclear image channel

From the drop down menu pick the nuclear channel image.

Step 2. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 3. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 4. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

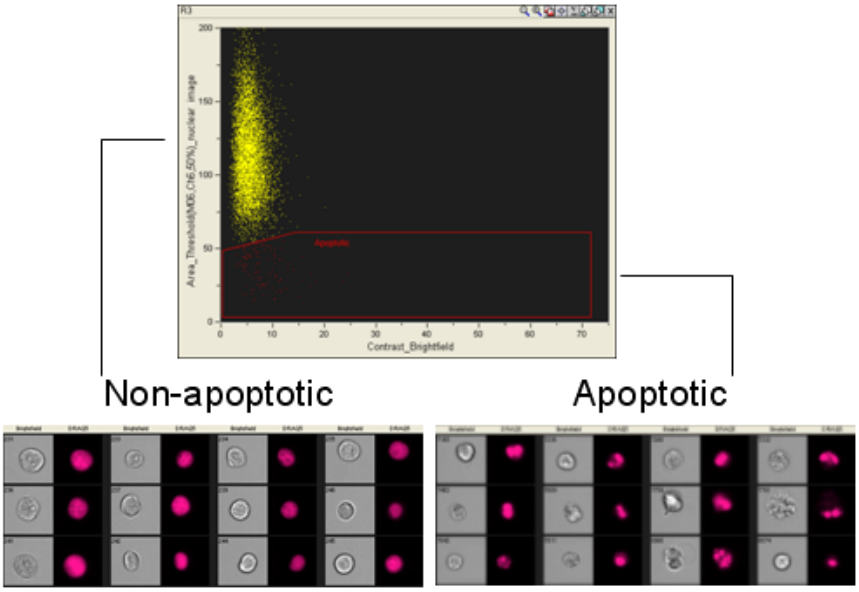
Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

Next Step. Optional - Select additional subpopulation marker(s) OR. Gate nucleated cells.

A histogram of the nuclear channel Intensity is added to the analysis area. Gate on the positive events.

Next Step . Gate apoptotic cells

The nucleated cells scatter plot of the brightfield Contrast versus the Area of the thresholded nucleus is added to the analysis area. Gate on the apoptotic cells with low nuclear area and high brightfield contrast.



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Cell Cycle - Mitosis Wizard

The cell cycle - mitosis wizard will guide you through the process of creating the features and graphs to analyze the cell cycle and identify mitotic events using the images of a nuclear dye.

To begin, double-click on Cell Cycle - Mitosis

Follow the instructions to analyze your file.

Step 1. Select the nuclear image channel

From the drop down menu pick the nuclear channel image.

Step 2. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 3. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 4. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

Next Step. Optional - Select additional subpopulation marker(s) OR. Gate G2/M cells.

A histogram of the nuclear channel Intensity is added to the analysis area. Gate on the G2/M population with 2n DNA.

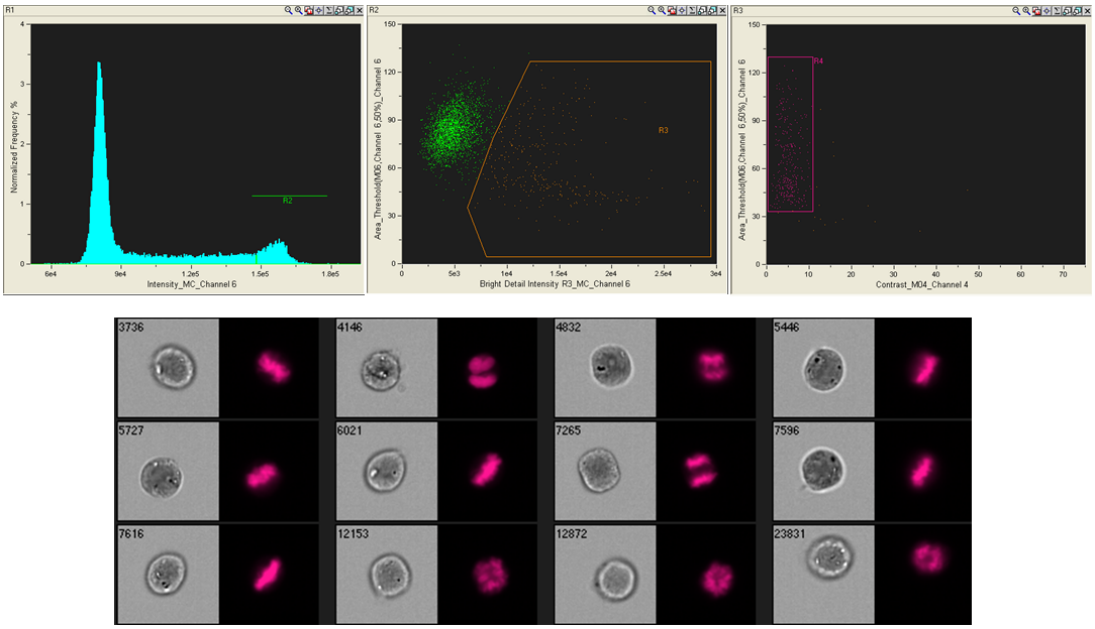
Next Step . Gate cells with condensed DNA

The G2/M cells scatter plot of the threshold Area versus Bright Detail Intensity of the nuclear image is added to the analysis area. Gate on the cells with condensed nuclear that have low nuclear area and high Bright Detail Intensity values These will include apoptotic cells which you can remove in the next step.

Next Step . Gate mitotic cells

The condensed DNA cells scatter plot of the brightfield Contrast versus the Area of the thresholded nucleus is added to the analysis area. Gate on mitotic events with low brightfield Contrast.

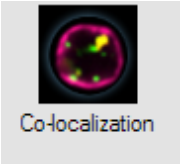
The final 3 plots are shown below:



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Co-localization Wizard

The co-localization wizard will guide you through the process of creating the features and graphs to measure the co-localization of two probes in any population of cells you identify.



To begin, double-click on Co-localization

Follow the instructions to analyze your file.

Step 1. Select the co-localization image channels

From the drop down menus pick the two image channels that contain the co-localizing probes.

Step 2. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 3. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 4. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

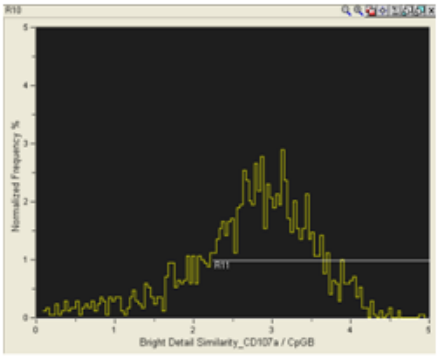
Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

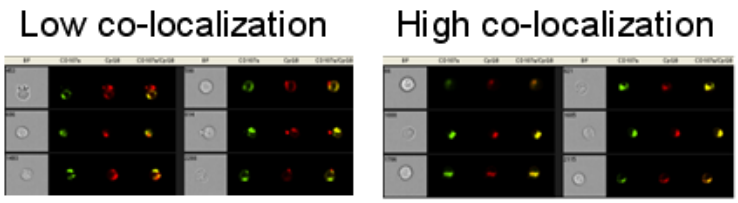
Next Step. Gate double positives

A scatter plot of the last gated (or selected) population of the Intensity values for the co-localization channels is added to the analysis area. Draw a region around the double positive cells for the co-localizing probes.

Next Step. Gate co-localized events

A histogram of Bright Detail Similarity R3 for the double positive population is added to the analysis area. Draw a region to gate on co-localized events.



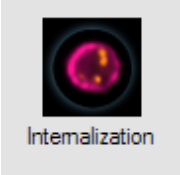


For a more thorough explanation of the **Bright Detail Similarity** feature see Bright Detail Similarity R3 Feature

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Internalization Wizard

This wizard will create an analysis template for measuring the internalization of a probe in any population of cells you identify.



To begin, double-click on Internalization

Follow the instructions to analyze your file.

Step 1. Select the internalization image channels

From the drop down menus pick the cell image, the channel that defines the cell surface, and the internalizing probe channel.

Step 2. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 3. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 4. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

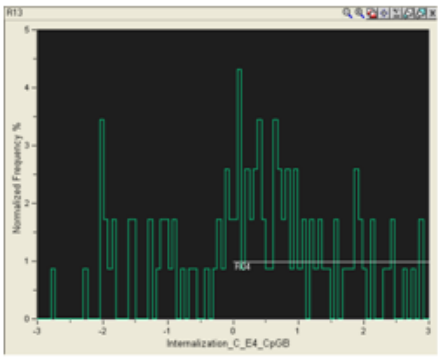
Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

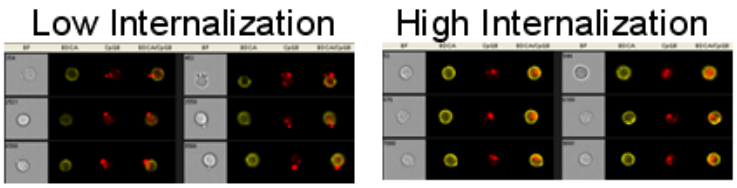
Next Step. Optional - Select additional subpopulation marker(s) OR. Gate internalization positives

A scatter plot of Max Pixel versus Intensity for the internalizing probe is added to the analysis area. Draw a region to include the positive cells.

Next Step. Gate internalization events

A histogram of the Internalization feature for the positive cells is added to the analysis area. Draw a region to include the cells with high internalization. The example below shows the internalization of labeled CpG (red).





For a more thorough explanation of the **Internalization** feature see[Internalization FeatureInternalization Feature]

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Nuclear Localization Wizard

This wizard will create an analysis template for measuring the nuclear localization of a probe in any population of cells you identify.

To begin, double-click on Nuclear Localization

Follow the instructions to analyze your file.

Step 1. Select the translocation image channels

From the drop down menus pick the nuclear image channel and the translocating probe image channel.

Step 2. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 3. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 4. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

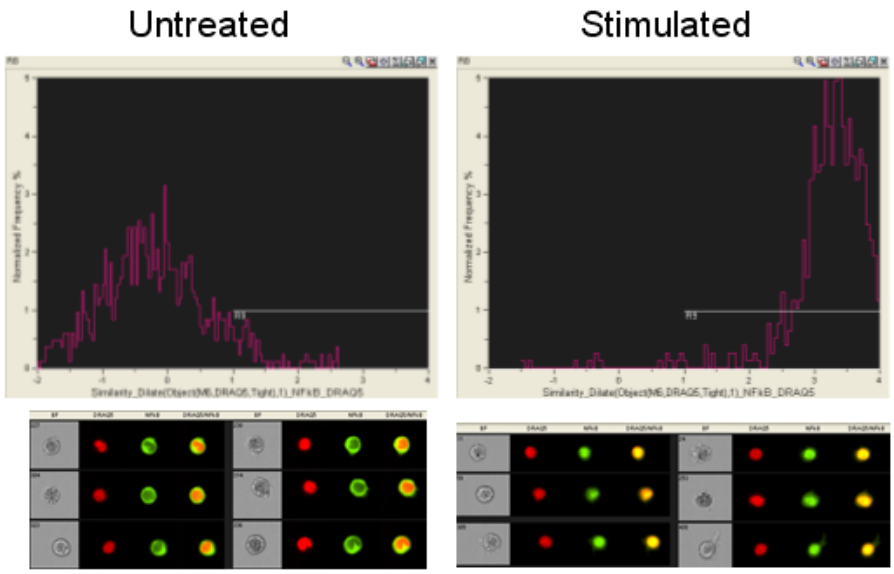
Next Step. Gate double positives

A scatter plot of the last gated (or selected) population of the Intensity values for the nuclear image and the translocating probe image is added to the analysis area. Draw a region around the double positive cells.

Next Step. Gate translocated events

A histogram of Similarity of the double positive cells is added to the analysis area. Draw a region to include the cells with translocation. Note that for a normally distributed population you may want to report the RD of the double positive population in a treated versus untreated sample instead of the percentage gated.

Nuclear localization of a probe is measured using the Similarity feature in the final graph presented in the wizard. The example shown here is of THP1 cells stimulated with 1 ug LPS for 90 minutes and stained with DRAQ5 (red) and NFkB (green) to measure the nuclear localization of the NFkB.

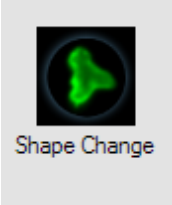


For a more thorough explanation of the **Similarity** feature see Similarity Feature .

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Shape Change Wizard

This wizard will create an analysis template for measuring the shape (circularity) of any population of cells you identify.



To begin, double-click on Shape Change

Follow the instructions to open and analyze your file.

Step 1. Select the cell morphology image channel

From the drop down menu pick the channel for the cell image.

Step 2. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 3. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 4. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

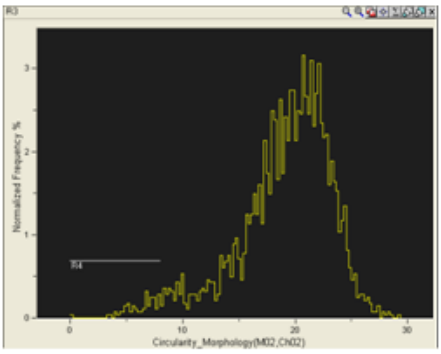
Next Step. Gate fluorescence positives

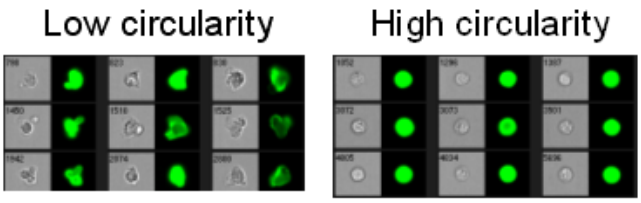
A histogram of the last gated (or selected) population of the Intensity value for the cell image is added to the analysis area. Draw a region around the double positive cells. Note that this step is skipped if the cell image channel chosen is brightfield.

Next Step. Gate shape-changed events

A histogram of Circularity of the last gated population is added to the analysis area. Draw a region to include the cells with low circularity scores.

Shape change is measured in the final graph presented in the wizard. Cells with low circularity scores have a highly variable radius. In this example monocytes in whole blood were stained with CD14 (green).



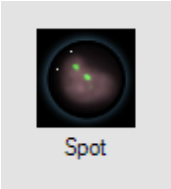


For a more thorough explanation of the **Circularity** feature see Circularity Feature .

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Spot Wizard

This wizard will create an analysis template for measuring texture based on spot counting. If the low and high spot count data are in separate data files merge the files together before beginning.



To begin, double-click on Spot

Follow the instructions to open and analyze your file.

Step 1. Gate cells in best focus

A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.

Step 2. Gate single cells

A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquistion.

Step 3. Optional

Answer Yes if you want to define subpopulations in your experiment.

Select subpopulation marker(s)

Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.

Next Step. Gate subpopulations step number sequence is dependent on the number of times the subpopulation marker step is taken.

Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

Next Step. Select the spot image channel

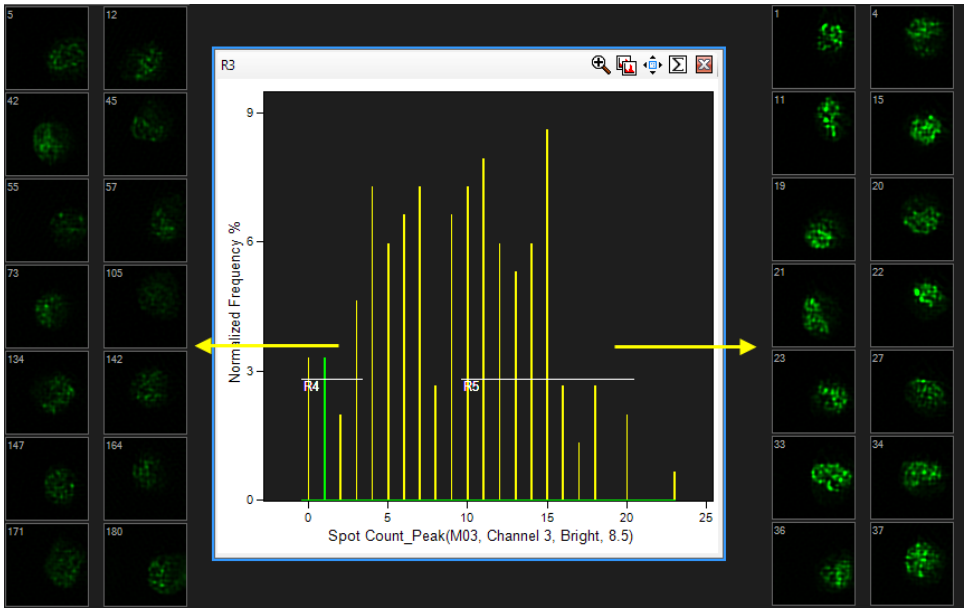
From the drop down menu choose the image channel for the spot counting.

Next Step. Assign truth populations

From the drop down menus select two truth populations, one with high and one with low spot count. To create the truth populations, either use the tagging tools or gate the cells of interest.

Next Step. Gate spot events

A histogram of the Spot Count feature for the last gated population is added to the analysis area. Regions have been drawn that include the truth populations. Adjust the regions as necessary. Note that you may want to adjust your truth populations and repeat the wizard after looking at the images and validating the spot counts.



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area and a statistics definition is added to the template. To view the definition choose **Define Statistics Report** from the Reports menu.

Building Blocks

Building blocks may be used to create a graph for finding single cells, focused cells or positive cells based on Intensity. The building blocks are shortcuts to creating a graph that provide a limited list of relevant features with set X and Y axis scales set for the graph. For more information on creating graphs see Creating Graphs.

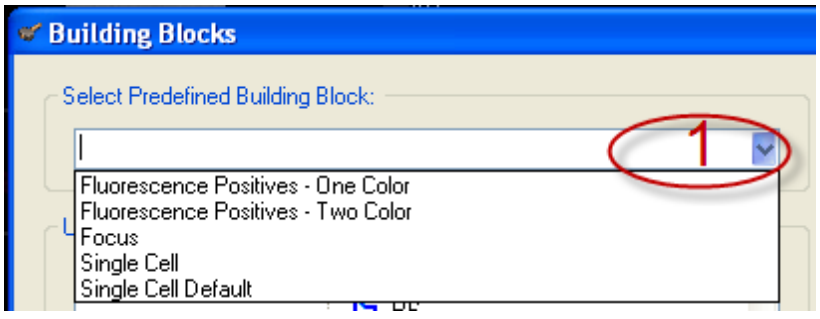
Table 1: Building blocks

Building Block	X axisFeatures	Y axis Features
Flourescence Positives - one color	Intensity_MC_ChX (for all channels)	
Flourescence Positives - two color	Intensity_MC_ChX (for all channels)	Intensity_MC_ChX (for all channels)
Focus	Gradient RMS_MX_ChX (for all channels) Note: Gradient RMS of brightfield is default	
Single Cell	Area_brightfield (default) Area_scatter Intensity_MC_ChX (for all channels)	Aspect Ratio_brightfield (default) Aspect Ratio Intensity_MX_ChX (for all fluorescence channels) Intensity_scatter
Single Cell Default	Area_brightfield	Aspect Ratio_brightfield
Size/SSC	Area_brightfield	Intensity scatter

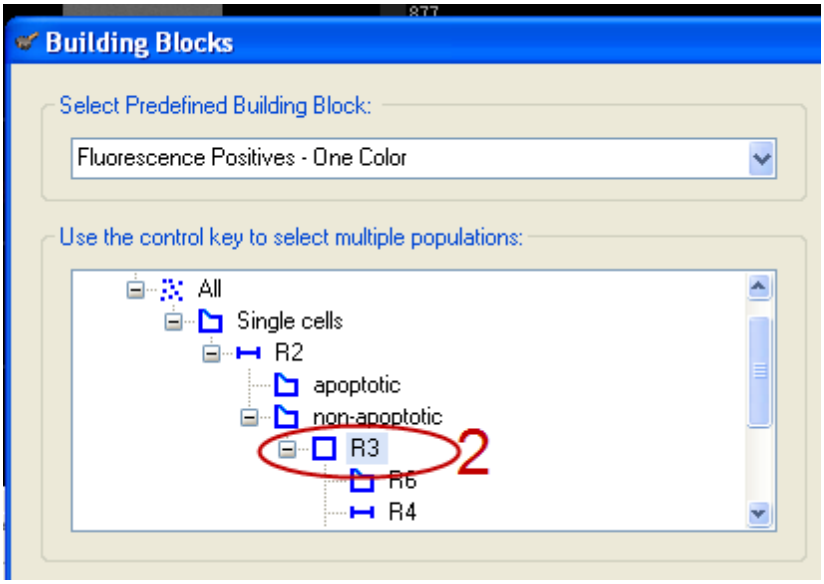
To begin, choose Building Blocks from the Guided Analysis Menu or click on the Building Blocks icon in the analysis area toolbar.

The Building Blocks window opens. This window is used to define a graph with a specified set of features available depending on the purpose of the graph.

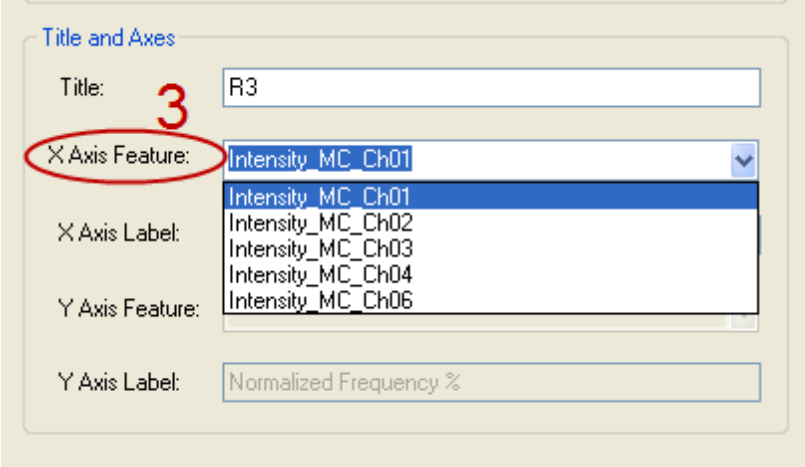
1 Choose the specific Building Block from the drop-down menu.



2 Choose the population(s) to graph.



3 Choose the X Axis Feature and the Y Axis feature, if applicable.



4 Click OK.

5 The graph is added to the analysis area.

Advanced Analysis

Opening data files

Saving Data Files

Overview of Compensation

Creating a New Compensation Matrix File

Viewing Sample Information

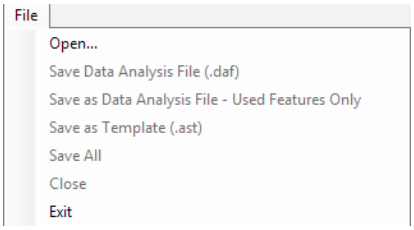
Merging Data Files

Creating new data files from populations

Batch Processing

Opening data files

Use the **File** menu, which is shown in the following figure, to open, save, and close image and analysis files and to quit the IDEAS application. Alternatively, you may open a data file by drag and drop into an open IDEAS window. Muliple data files can be open in one instance of the IDEAS application.



Opening a .rif file

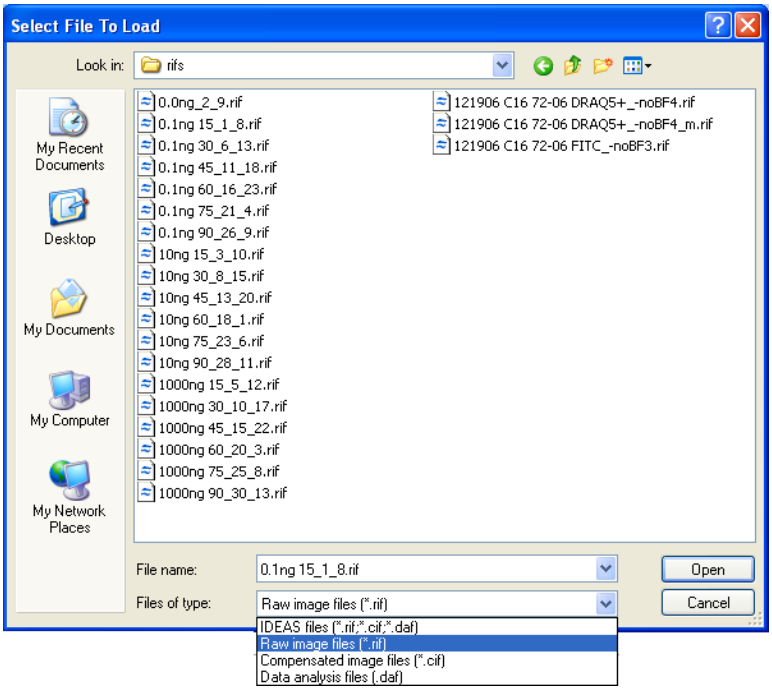
A .rif file is opened when there is new data and the IDEAS application needs to apply corrections. When opening a .rif file, the IDEAS application corrects each image for the spatial alignment between channels, camera background normalization, flow speed, and brightfield gain normalization. If you want fluorescence compensation to correct for spectral overlap, you must create or choose a compensation matrix at this time by using the control files that were collected for a particular experiment. If a FlowSight data file was acquired with a compensation matrix, that matrix will be used by default. For more information on compensation see Creating a New Compensation Matrix File. The application performs the corrections by using calibration information that was saved to the .rif file during acquisition.

To open a .rif file

To use a wizard to do this see Open File Wizard:, otherwise:

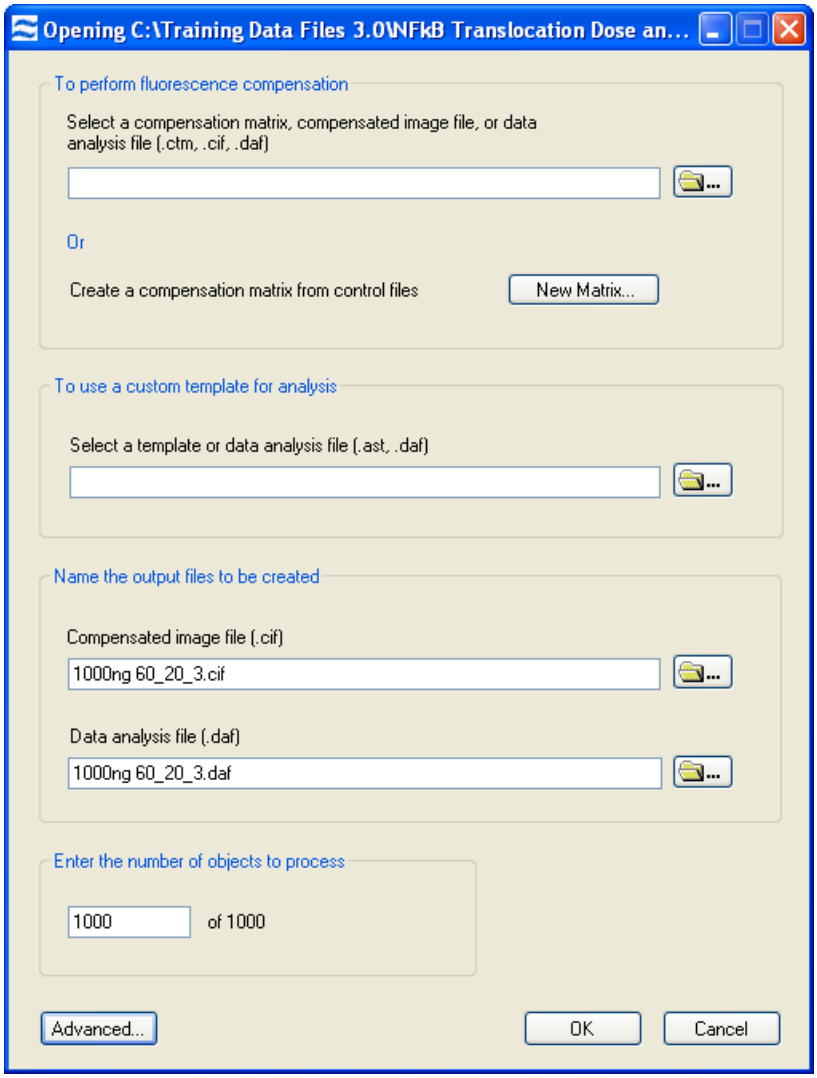
1 From the File menu, choose **Open** or drag the file into the IDEAS window.
2 Select the .rif file that you want in the **Select File To Load** window.

Tip: while browsing for the file to open you can limit the type of file shown in the window to .rif files.



In the next window you will:

- Choose a compensation matrix
- Choose a template
- Name the output files
- Choose the number of events to process


Opening C:\Training Data Files 3.0\NFkB Translocation Dose an...
To perform fluorescence compensation
Select a compensation matrix, compensated image file, or data analysis file (.ctm, .cif, .daf)
Or
Create a compensation matrix from control files
New Matrix...
To use a custom template for analysis
Select a template or data analysis file (.ast, .daf)
Name the output files to be created
Compensated image file (.cif)
1000ng 60_20_3.cif
Data analysis file (.daf)
1000ng 60_20_3.daf
Enter the number of objects to process
1000
of 1000
Advanced...
OK
Cancel

3 Click the folder next to **Select a compensation matrix, compensated image file, or data analysis file (.ctm, .cif, .daf)** field to choose the matrix that was generated from the controls used for the experiment. If the .rif file contains a compensation matrix used during acquisition it will be entered into this box. If you leave it blank, the default compensation matrix will be used, but this is not recommended unless you do not want to compensate your data.

- If a compensation matrix for the experiment has not been made, click **New Matrix**. For more information on creating a compensation matrix see Creating a New Compensation Matrix File.

4 In the **Select a template or data analysis file (.ast, .daf)** field, select a template file to load by clicking the folder and browsing for the file. If left blank, the Default template with the basic features, masks, and settings will be used. FlowSight files use the acquisition template as the default.

5 Name the output files with a new name, if necessary.

6 You may change the number of objects to load in the box under **Enter the number of objects to process**. The default value is the number of objects in the file.

Tip: you can select a smaller number than the maximum if you have a large number of objects to load. This helps save time for creating a template file. The IDEAS application randomly loads the specified number of objects within the file.

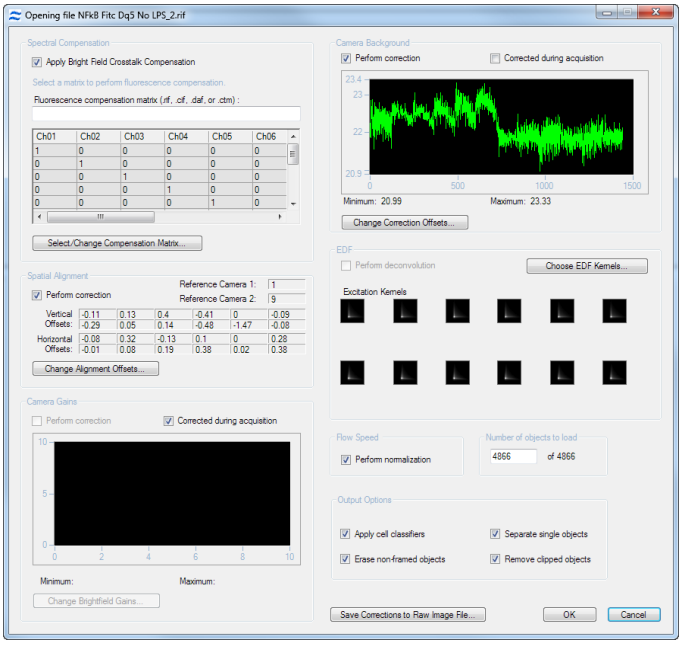
7 Click **OK**.

The application then creates the .cif and .daf files and the .daf file is loaded into the Image Analysis area.

Note: Most often, the defaults will be adequate. For some older data files, you may need to provide control files for certain settings. For assistance call Amnis application support.

- To view the corrections that will be applied to the .rif file, click **Advanced** within the Opening a .rif file window.

  The **Opening file** window appears.



- Make any changes to the corrections that you need, and then click **OK**.

Opening a .cif file

A .cif file is generated when corrections are applied to a .rif file, as described in Overview of the Data File Types. When opening a .cif file, the IDEAS application calculates feature values and creates a .daf file to display images and graphs.

When opening a .cif file, an analysis template is selected. The template provides the initial characteristics of the analysis. Opening the .cif file causes the IDEAS application to calculate feature values and to use populations, graphs, and image viewing settings to display the cells as defined by the template.

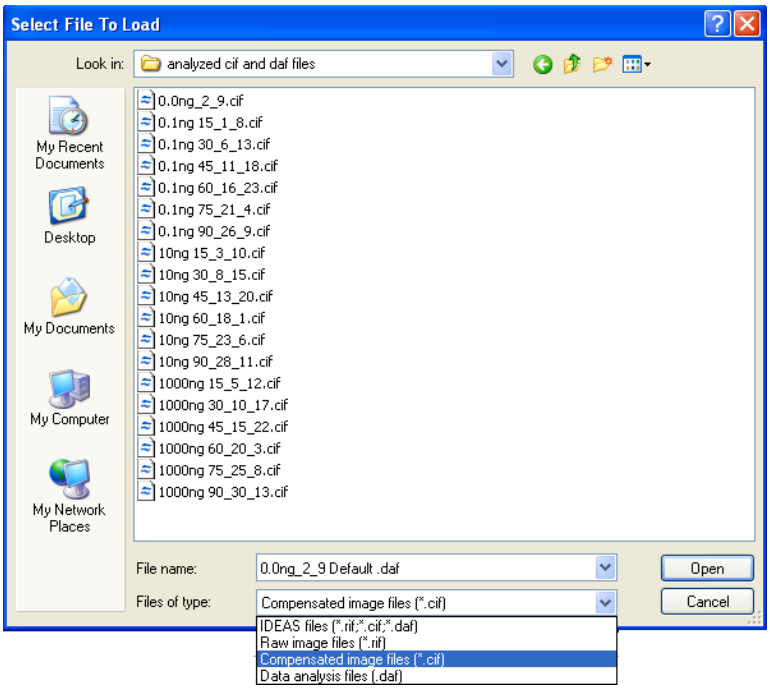
To open a .cif file

To use a wizard to do this see Open File Wizard:, otherwise:

1 From the File menu, choose **Open** or drag the file into the IDEAS window.

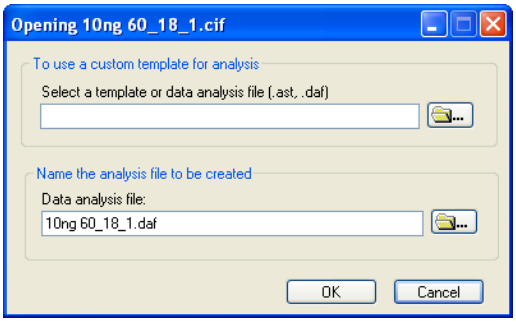
2 Select the .cif file that you want in the Select File To Load window.

Tip: while browsing for the file to open you can limit the type of file shown in the window to .cif.



In the next window you will:

- Choose a template
- Name the output file


Opening 10ng 60_18_1.cif
To use a custom template for analysis
Select a template or data analysis file (.ast, .daf)
Name the analysis file to be created
Data analysis file:
10ng 60_18_1.daf
OK
Cancel

3 Click the folder next to **Select a template or data analysis file (.ast, .daf)** and choose the template to use for analysis. If left blank, the IDEAS application will use a default template. However, it is useful to create and save your own templates for specific experimental procedures.

4 Change the **Data analysis file** name, if necessary. The default name matches the name of the .cif.

5 Click **OK**.

During the opening of a .cif file, the IDEAS application calculates the values of the features that are defined in the template you selected. The progress is shown by a progress bar. After the application has successfully opened the .cif file, the .daf file is saved.

See also: Saving Data Files.

Opening a .daf file

A .daf file contains the calculated feature values so that they will not need to be recalculated, as described in Overview of the Data File Types. To open a .daf file, the IDEAS application requires the .cif file to reside in the same directory. The .daf file does not contain any image data, so you can think of the .cif file as the database that contains the imagery. Because all of the feature values have been saved in it, the .daf file should open quickly.

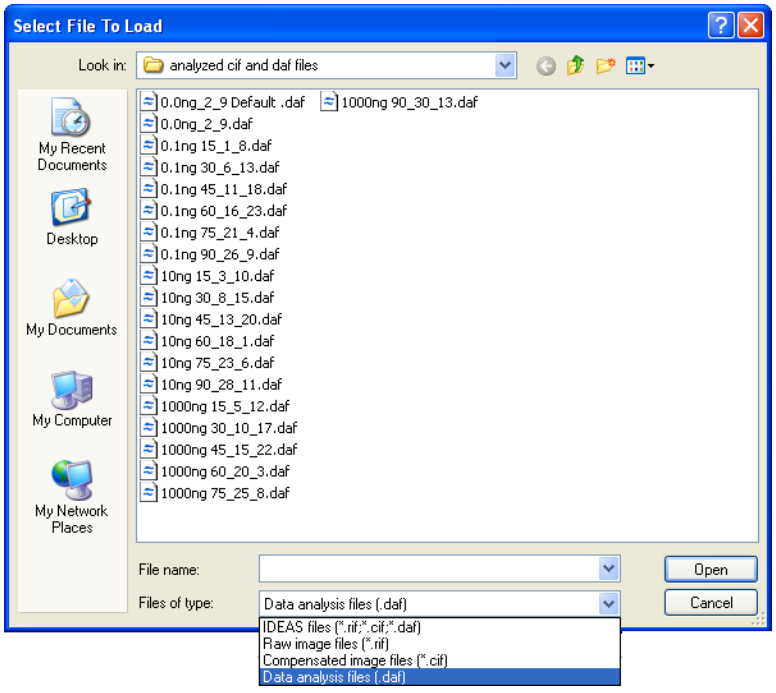
To open a .daf file

To use a wizard to do this see Open File Wizard:, otherwise:

1 From the File menu, choose **Open** or drag the file into the IDEAS window.

2 Select the .daf file that you want in the Select File To Load window.

Tip: while browsing for the file to open you can limit the type of file shown in the window to .daf.



The progress is shown by a progress bar. The state of the IDEAS application is restored to what it was when the .daf file was saved.

Saving Data Files

Data files are saved at several stages of analysis. Raw image files are saved during data acquisition, by merging multiple .rif files or by creating new files from populations. Compensated image files and Data analysis files are saved when opening .rif files, merging multiple .cif files, or when running a batch analysis. The IDEAS application also saves other types of files that are used for data correction and presentation. Template files (.ast) save the structure of an analysis and compensation matrix files (.ctm) save the compensation matrices.

Application Defaults are set that direct the files into specific folders and can be viewed or changed by the user. See Viewing and Changing the Application Defaults for more information.

Saving a Data Analysis File (.daf)

A .daf file contains a snapshot of an analysis as described in Overview of the Data File Types. Saving the analysis as a .daf file allows you to recall that analysis simply by opening the file. When you quit the IDEAS application, you are always prompted to save changes to a .daf file. You can also save changes from the File menu. Remember that the .daf file does not contain image information, so opening the .daf file requires the related .cif file to reside in the same directory.

To save a .daf file

1 On the **File** menu, click **Save as Data Analysis File (.daf)**.

2 Enter a file name. Note that the default directory is the one where the .cif file was saved.

If you select an existing file name, a warning appears that asks you to verify the overwriting of the existing file.

3 Click **Save**.

The data is now ready for analysis. You can create graphs, view imagery, and display feature values and statistics. After you have defined an analytical procedure in the .daf file, you can save the file as a template, which allows you to use the procedure for analyzing other files. Refer to Overview of the Data Analysis Tools for more information.

option: Saving a data analysis file with only the feature values used.

When you want to reduce the size of a data analysis file you may save the .daf with only the features that are used for analysis of statitics or graphs.

On the File menu, click Save as Data Analysis File - Used Features Only. and follow the instructions 2-3 above.

Saving a Compensated Image File (.cif)

The IDEAS application creates and saves a .cif file when a .rif file is opened. By default, the application names the .cif file with the same name that the .rif file has, replacing the .rif extension with .cif. The application also places the .cif file in the

same directory as the .rif file. The .cif file will be larger than the .rif file because the .cif file contains masking information as well as corrected and/or compensated images.

Saving a Template (.ast)

Saving an analysis as a template allows you to apply the structure of the analysis to other .cif files. Save a template file after saving a .daf file. A template includes all graph definitions, Image Gallery settings, feature definitions, and statistics settings. No data are saved in a template. Therefore, selected images and populations that are dependent on specific objects, such as tagged populations, are not saved.

To save a template

1 On the **File** menu, click **Save As Template File (.ast)**.

A **Save File** dialog box appears.

2 Enter the name of the file to save.

3 Click **Save**.

If you select an existing file name, a warning appears that asks you to verify the overwriting of the existing file.

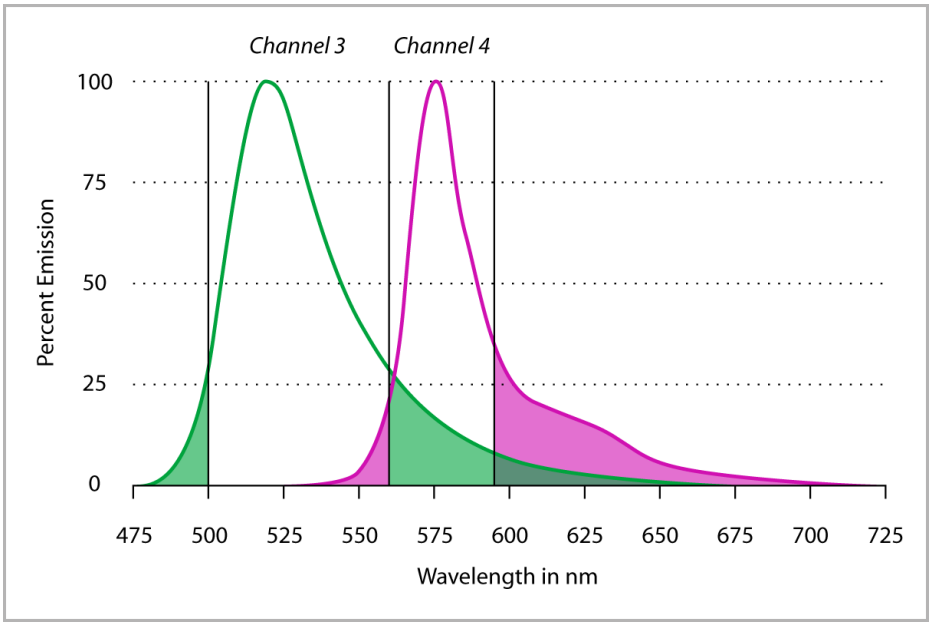
Tip: You can change the default template directory in the menu **Analysis** > **Application Defaults**.

Overview of Compensation

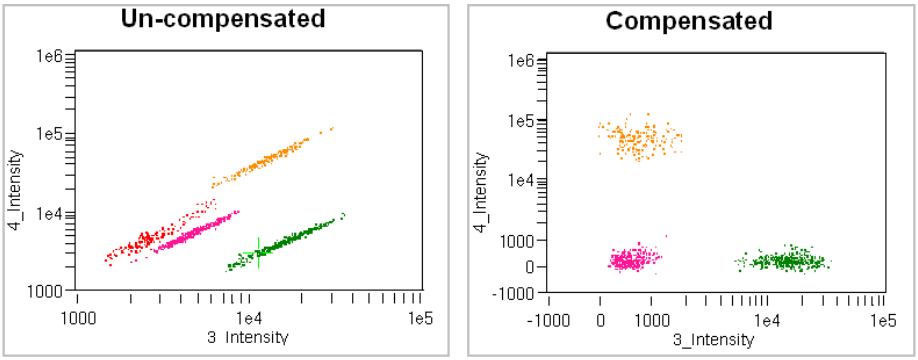
Spectral compensation corrects imagery for fluorescence that leaks into nearby channels so that you may accurately depict the correct amount of fluorescence in each cell image.

For example, the light from one fluorochrome may appear primarily in channel 3, but some of the light from this fluorochrome may appear in channel 4 as well, because the emission spectrum of the probe extends beyond the channel 3 spectral bandwidth. The light from a second fluorochrome may appear primarily in channel 4 but, unless you subtract the light emitted by the first fluorochrome into channel 4, you cannot generate images that accurately represent the distribution of the second fluorochrome.

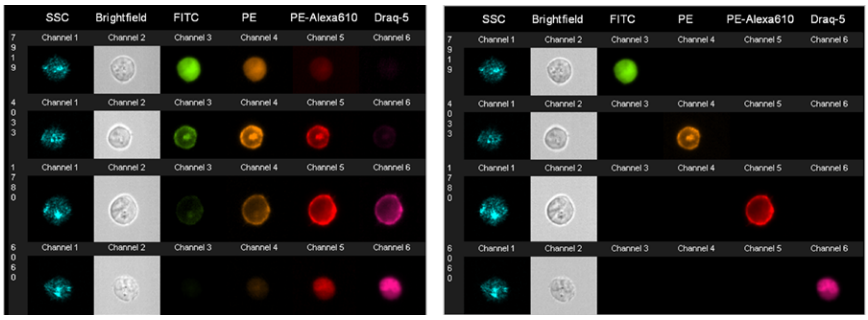
Emmission Spectra for two fluorochromes:



Below is an example of cells stained with two fluorochromes independently and run together as one sample. Intensity scatter plots and images are shown uncompensated and compensated. Image compensation is performed on a pixel by pixel basis.



Uncompensated Compensated



The IDEAS application builds a matrix of compensation values by using one or more control files. A control file contains cells stained with one fluorochrome. Because it is critical that matrix values be calculated from intensities derived from a sole source of light, control files are collected without brightfield illumination, or scatter. The IDEAS application performs brightfield compensation when it loads a .rif file. The process of creating the compensation matrix is described in the next section.

Creating a New Compensation Matrix File

The compensation matrix is a table of coefficients. The IDEAS application uses this table to place the detected light that is displayed in each image into the proper channels, on a pixel-by-pixel basis. The coefficients are normalized to 1. Each coefficient represents the normalized amount of the leakage of the fluorochrome into the other channels.

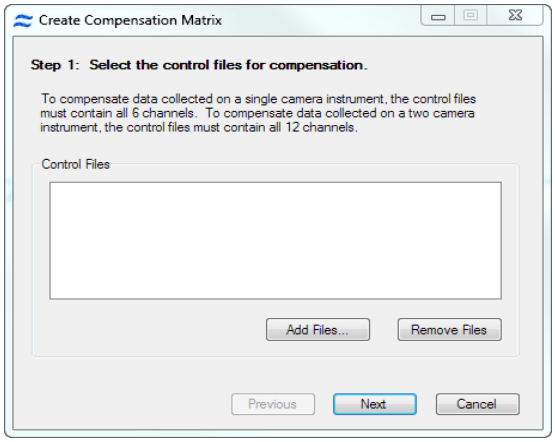
The default matrix, which is used if no compensation matrix is chosen, is the identity matrix, shown below.

	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0	0	0	0	0	0	0	0	0	0	0
Ch02	0	1	0	0	0	0	0	0	0	0	0	0
Ch03	0	0	1	0	0	0	0	0	0	0	0	0
Ch04	0	0	0	1	0	0	0	0	0	0	0	0
Ch05	0	0	0	0	1	0	0	0	0	0	0	0
Ch06	0	0	0	0	0	1	0	0	0	0	0	0
Ch07	0	0	0	0	0	0	1	0	0	0	0	0
Ch08	0	0	0	0	0	0	0	1	0	0	0	0
Ch09	0	0	0	0	0	0	0	0	1	0	0	0
Ch10	0	0	0	0	0	0	0	0	0	1	0	0
Ch11	0	0	0	0	0	0	0	0	0	0	1	0
Ch12	0	0	0	0	0	0	0	0	0	0	0	1

To generate a new compensation Matrix file

1 Start the Compensation Wizard in one of two ways:
   - Click the **New Matrix** button when opening a .rif file
   - **OR** select **Compensation>Create New Matrix** .

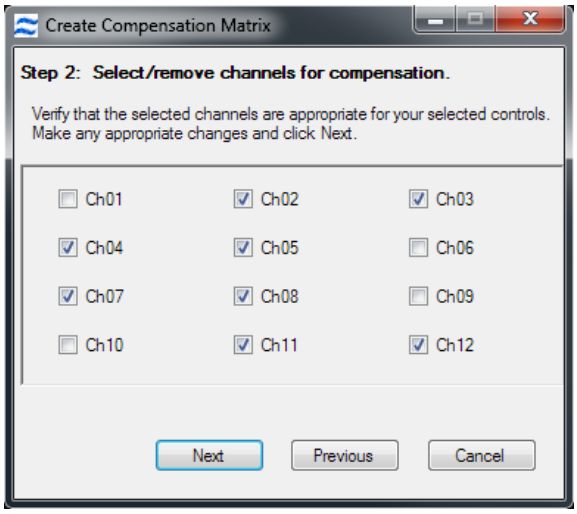
   The compensation wizard opens to **Step 1**:


Create Compensation Matrix
Step 1: Select the control files for compensation.
To compensate data collected on a single camera instrument, the control files must contain all 6 channels. To compensate data collected on a two camera instrument, the control files must contain all 12 channels.
Control Files
Add Files...
Remove Files
Previous
Next
Cancel

2 Add compensation control files by clicking Add Files and browsing for the control files for the experiment. The files will have the suffix no-BF. Hold down the control key to select multiple files at once.

3 When all of the control files for the experiment have been added to the list, click **Next**. The control file(s) are merged and loaded.

4 **Step 2**: Verify the channels for each control in the experiment by checking the channel boxes.



The following tables are provided as a guide for each instrument configuration.

Table 1: First generation ImageStream (IS100)

Ch 1	Ch 2	Ch3	Ch 4	Ch 5	
470-500nm	**400-470nm**	**500-560nm**	**560-595nm**	**595-660nm**	**660-735nm**
Scatter	DAPI	FITC	PE	7-AAD	PE-Cy5

Table 2: ImageStream^X- 1 camera

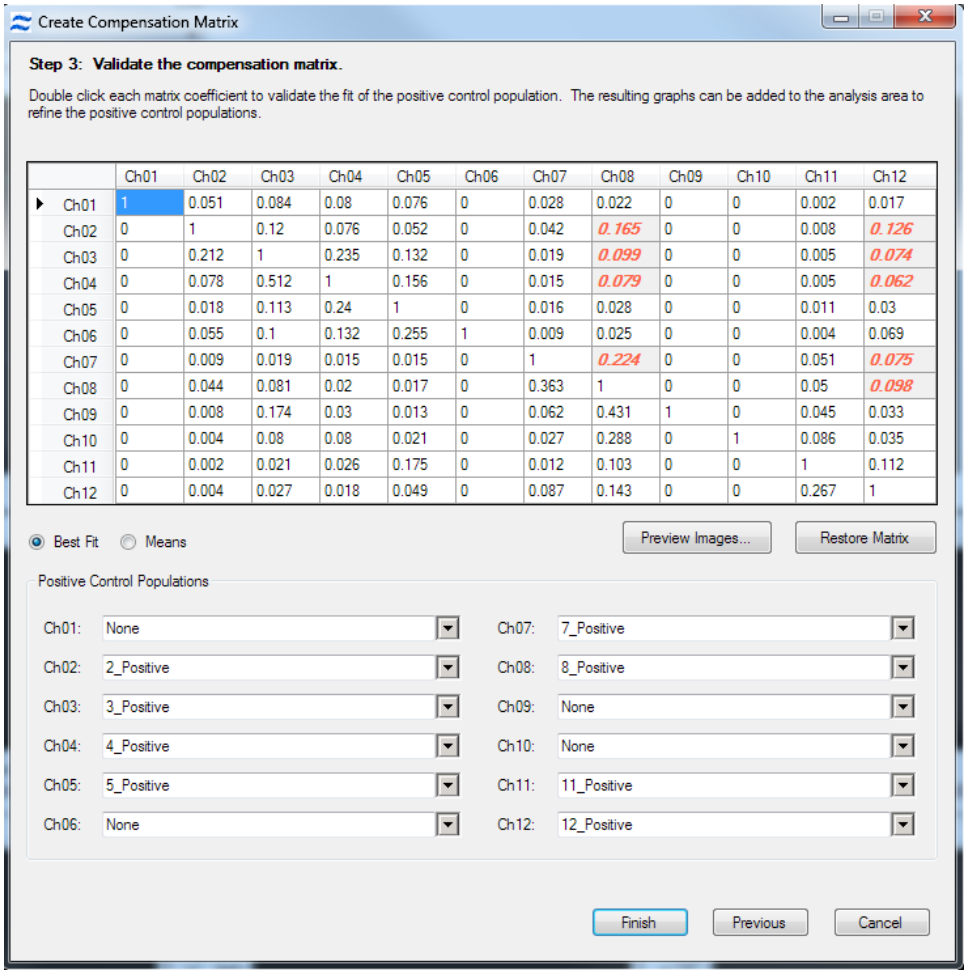
					Ch 6
430-505nm	**505-560nm**	**560-595nm**	**595-660nm**	**660-745nm**	**745-800nm**
DAPI	FITC	PE	PE-Tex-asRed	AF647	APC-Cy7

Table 3: ImageStream^X- 2 camera or FlowSight

					Ch 6
430-480nm	**480-560nm**	**560-595nm**	**595-660nm**	**660-745nm**	**745-800nm**
BF	FITC	PE	PE-Tex-asRed	PE-Cy5	PE-Cy7
			Ch 10	Ch 11	Ch 12
430-505nm	**505-570nm**	**570-595nm**	**595-660nm**	**660-745nm**	**745-800nm**
DAPI	Pacific Orange	BF	Texas Red	AF647	APC-Cy7

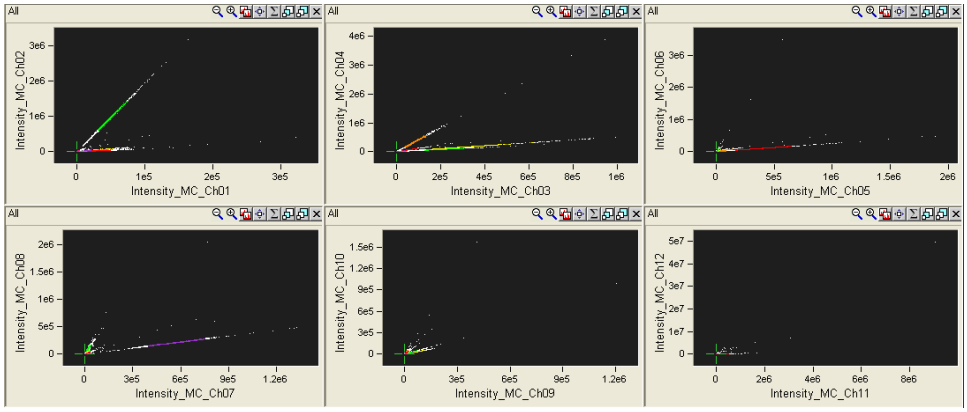
5 Background and spatial offset corrections are performed, the imagery is displayed, bivariate plots of adjacent channels Intensity are added to the analysis area and the compensation matrix values are computed and displayed in a table.

Example:



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.051	0.084	0.08	0.076	0	0.028	0.022	0	0	0.002	0.017
Ch02	0	1	0.12	0.076	0.052	0	0.042	*0.165*	0	0	0.008	*0.126*
Ch03	0	0.212	1	0.235	0.132	0	0.019	*0.099*	0	0	0.005	*0.074*
Ch04	0	0.078	0.512	1	0.156	0	0.015	*0.079*	0	0	0.005	*0.062*
Ch05	0	0.018	0.113	0.24	1	0	0.016	0.028	0	0	0.011	0.03
Ch06	0	0.055	0.1	0.132	0.255	1	0.009	0.025	0	0	0.004	0.069
Ch07	0	0.009	0.019	0.015	0.015	0	1	*0.224*	0	0	0.051	*0.075*
Ch08	0	0.044	0.081	0.02	0.017	0	0.363	1	0	0	0.05	*0.098*
Ch09	0	0.008	0.174	0.03	0.013	0	0.062	0.431	1	0	0.045	0.033
Ch10	0	0.004	0.08	0.08	0.021	0	0.027	0.288	0	1	0.086	0.035
Ch11	0	0.002	0.021	0.026	0.175	0	0.012	0.103	0	0	1	0.112
Ch12	0	0.004	0.027	0.018	0.049	0	0.087	0.143	0	0	0.267	1

The Positive Control Populations are shown in the graphs below.



6 In **Step 3**, choose one of two methods for calculating the coefficients.

- The **Best Fit** method is used for objects such as cells where intensities vary.
- The **Means** method is used for objects such as beads that have only slight variations in intensity.

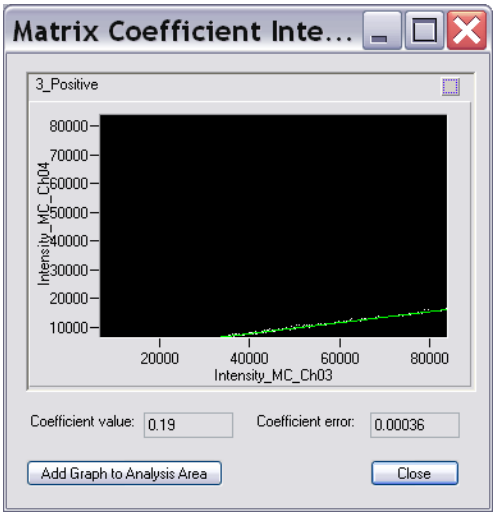
- For each fluorochrome, the application automatically identifies a positive control population, excluding the brightest and dimmest objects, and assigns it to the proper channel.

7 Inspect the matrix values in the table of coefficients.

Coefficients should always be less than 1, and decrease from the assigned channel. In other words, leakage should be greater in the channel nearest to the assigned channel. Fluorescence always extends in the long-wavelength direction from the exciting light.

- Verify that no coefficients are larger than 1.
- Verify that, in a column corresponding to a fluorochrome, the coefficients decrease from the assigned channel.
- Verify that the coefficient is greater in the channel below the 1 in the table than the value above the 1 in the table. Verify that these coefficients also decrease in subsequent channels below the 1.
- Verify that there are no changes from the identity matrix in the columns where there are no fluorochromes, including the scatter and brightfield channels. If necessary, the column can be set to the identity values by double-clicking on the heading.
- Inspect the coefficients in the matrix by double-clicking on the coefficient. Coefficients highlighted by red have errors greater than 1%.

  A graph representing the coefficient appears. The population potted in the graph is the positive control population of the column of the coefficient. The X Axis represents the intensity in the assigned channel of the fluorochrome. The Y Axis represents the intensity in the channel of leakage. The coefficent value and error are also displayed.

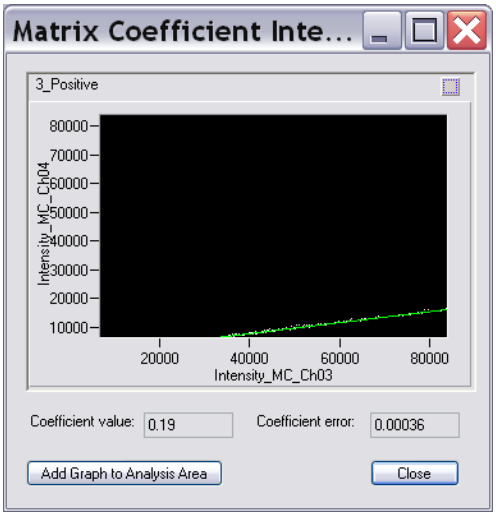


- You can use the automatically generated control populations as they are, or you can refine them and create different populations by using the region tools. See the option below to remove objects from the population. By default, the populations are named 3_Positive, 5_Positive, and so on. You can view the populations in the Image Gallery. Some populations may be empty.
- To choose a different population, use the arrow and select the population from the list. The hierarchical relationship is shown in the population list. Assign

populations only to the channels that correspond to the fluorochromes used in the experiment.

- If you want to clear a column, double click on the channel heading.
- If needed, you can create new scatter plots by using the Analysis Area toolbar. For example, a 4_Intensity versus 5_Intensity plot may be useful. See Creating Graphs for more information.
- The slope of the line on the plot is the coefficient in the matrix.

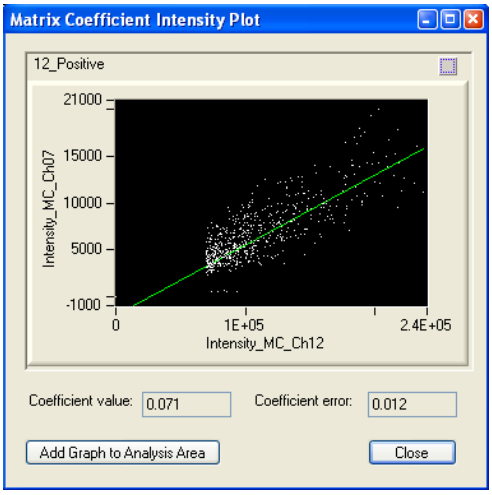
8 If objects in the population exist that are outliers, they should most likely be removed from the positive population within the compensation matrix by the following optional steps.



- The slope of the line on the plot is the coefficient in the matrix.
- If objects in the population exist that are outliers, they should most likely be removed from the positive population within the compensation matrix by the following optional steps.

Option: Remove Objects from the Population

1 Within Step 2 of the compensation wizard, double-click the coefficient to display the intensity plot.



2 If you notice outliers, click **Add Graph to Analysis Area**.

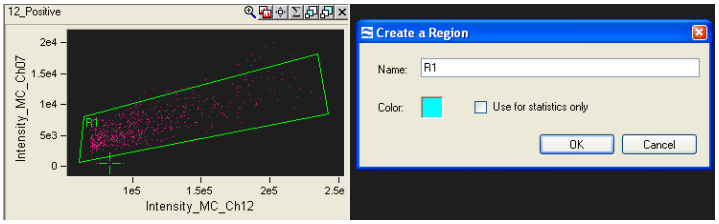
The plot populates in the Analysis Area.

3 Return to the Analysis Area and use the region tools to draw a new region on the plot that defines a new positive control population, excluding the outliers. Refer to Creating Regions on Graphs for more information.

- Create a new region to exclude outliers.

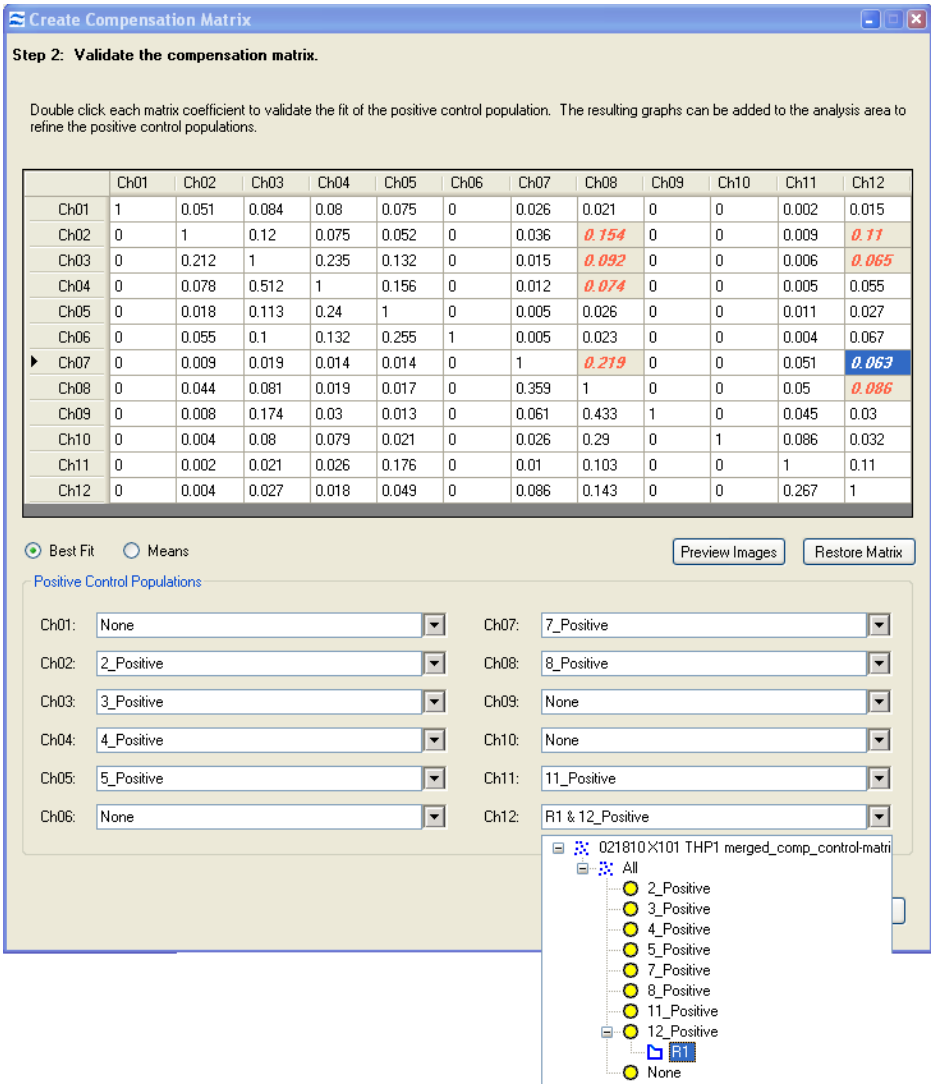
Click the Resize and Zoom buttons on the graph toolbar to more clearly see the population of interest. Using one of the region buttons on the toolbar, draw a region that contains only the cells you want to use for determining compensation. You can click a point on the graph and view the image to help you decide where the region boundaries should be.

In the example below, the Polygon Region tool was selected to draw a border around a selection of cells. Clicking within the graph anchors the line and double-clicking completes the region.



For more information, refer to Creating Regions on Graphs.

4 Assign the new population to the appropriate channel by using the **Positive Control Populations** list for that channel.



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.051	0.084	0.08	0.075	0	0.026	0.021	0	0	0.002	0.015
Ch02	0	1	0.12	0.075	0.052	0	0.036	*0.154*	0	0	0.009	*0.11*
Ch03	0	0.212	1	0.235	0.132	0	0.015	*0.092*	0	0	0.006	*0.065*
Ch04	0	0.078	0.512	1	0.156	0	0.012	*0.074*	0	0	0.005	0.055
Ch05	0	0.018	0.113	0.24	1	0	0.005	0.026	0	0	0.011	0.027
Ch06	0	0.055	0.1	0.132	0.255	1	0.005	0.023	0	0	0.004	0.067
Ch07	0	0.009	0.019	0.014	0.014	0	1	*0.219*	0	0	0.051	*0.063*
Ch08	0	0.044	0.081	0.019	0.017	0	0.359	1	0	0	0.05	*0.086*
Ch09	0	0.008	0.174	0.03	0.013	0	0.061	0.433	1	0	0.045	0.03
Ch10	0	0.004	0.08	0.079	0.021	0	0.026	0.29	0	1	0.086	0.032
Ch11	0	0.002	0.021	0.026	0.176	0	0.01	0.103	0	0	1	0.11
Ch12	0	0.004	0.027	0.018	0.049	0	0.086	0.143	0	0	0.267	1

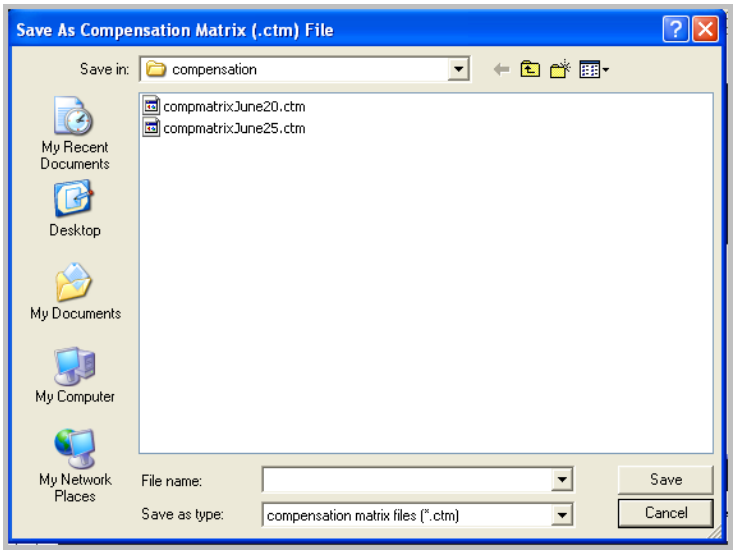
5 The coefficient value is automatically recalculated when a new population is selelcted.

6 Repeat these steps as required to redefine the coefficients.

7 Click Preview Images to view individual objects with corrections applied. Double click on an image to add it to the preview window. Note: the corrections are only applied to on-camera channels. For example, if the object is brightest in channel 3 on the first camera, only channels 1-6 are shown corrected for that object.

8 When the matrix appears satisfactory, click **Finish**.

9 Enter a name for the compensation matrix file (.ctm) and click **Save**



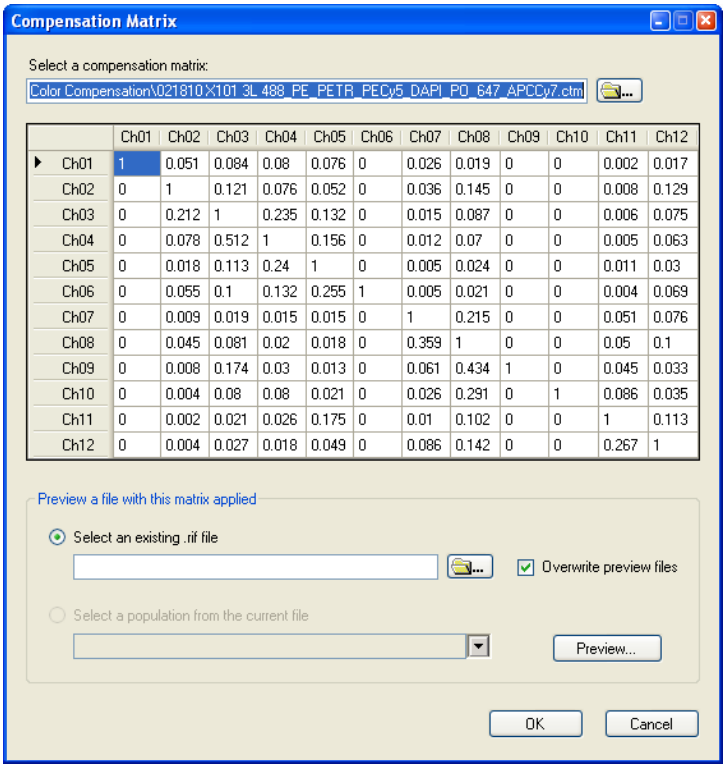
Note: The matrix is saved as a compensation matrix file (.ctm file). This file contains the compensation values and can be opened later for editing. To provide the values for fluorescence compensation, you select a .ctm file when opening a .rif file. See Opening a .rif file for more information.

Preview and edit a compensation matrix

A compensation matrix can be applied to a population or .rif file in a preview mode for editing a matrix.

To open a compensation Matrix

1 Select View/Edit Compensation Matrix from the Compensation menu to view, edit or preview the matrix on image data.Select the data file by clicking on the folder and then click Open. The matrix values are displayed in a table and may be edited.



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.051	0.084	0.08	0.076	0	0.026	0.019	0	0	0.002	0.017
Ch02	0	1	0.121	0.076	0.052	0	0.036	0.145	0	0	0.008	0.129
Ch03	0	0.212	1	0.235	0.132	0	0.015	0.087	0	0	0.006	0.075
Ch04	0	0.078	0.512	1	0.156	0	0.012	0.07	0	0	0.005	0.063
Ch05	0	0.018	0.113	0.24	1	0	0.005	0.024	0	0	0.011	0.03
Ch06	0	0.055	0.1	0.132	0.255	1	0.005	0.021	0	0	0.004	0.069
Ch07	0	0.009	0.019	0.015	0.015	0	1	0.215	0	0	0.051	0.076
Ch08	0	0.045	0.081	0.02	0.018	0	0.359	1	0	0	0.05	0.1
Ch09	0	0.008	0.174	0.03	0.013	0	0.061	0.434	1	0	0.045	0.033
Ch10	0	0.004	0.08	0.08	0.021	0	0.026	0.291	0	1	0.086	0.035
Ch11	0	0.002	0.021	0.026	0.175	0	0.01	0.102	0	0	1	0.113
Ch12	0	0.004	0.027	0.018	0.049	0	0.086	0.142	0	0	0.267	1

2 To preview the matrix on image data, browse for a file or select a population from the current file to preview and click Preview.

Note: It is recommended that you first create a small tagged population to preview compensation changes because previewing large populations requires a lot of memory and may be slow.

3 You may repeat editing the matrix and previewing until satisfied.

4 When done, click OK and save the matrix.

Merging Data Files

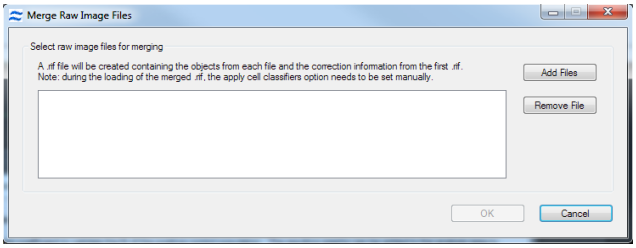
Merging Raw Image Files

You can merge .rif files together for analysis. This option is not available for basic FlowSight files without the Quantitative Imaging (QI) upgrade.

To merge .rif files

1 On the **Tools** menu, click **Merge .rif Files**.

The **Merge Raw Image Files** window appears.


Merge Raw Image Files
Select raw image files for merging
A .rif file will be created containing the objects from each file and the correction information from the first .rif.
Note: during the loading of the merged .rif, the apply cell classifiers option needs to be set manually.
Add Files
Remove File
OK
Cancel

2 To select the .rif files to merge, click **Add Files**.

The .rif file names appear in the list.

3 If you want to remove a file from the list, select it and then click **Remove File**.

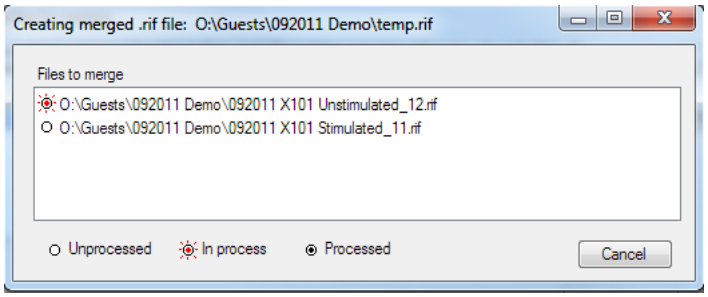
4 When the merge list is complete, click **OK**.

The **Save Merged Raw Image (.rif) File** dialog box appears.

5 Type a unique file name.

6 Click **Save**.

The Creating merged .rif file window appears.



When the merge is complete, the **Merged .rif Created** message appears.

7 Click **OK**.

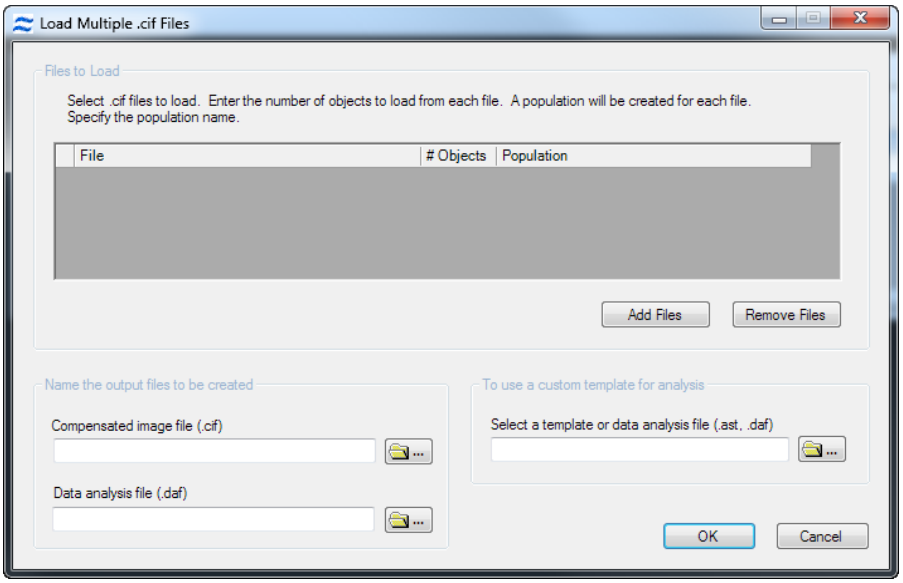
Note: The sample information will contain the classifier information for the first file in the merge list however the classifier is turned off when a merged file is loaded. To turn the classifier on manually, go to the Advanced panel on the open .rif window when opening a merged file.

Merging Compensated Image Files

You can merge .cif files together for analysis.This option is not available for basic FlowSight files without the Quantitative Imaging (QI) upgrade.

To merge .cif files

1 On the **Tools** menu, click **Merge .cif Files**.
The **Load Multiple .cif Files** window appears.


Load Multiple .cif Files
Files to Load
Select .cif files to load. Enter the number of objects to load from each file. A population will be created for each file.
Specify the population name.
File
Objects
Population
Add Files
Remove Files
Name the output files to be created
Compensated image file (.cif)
Data analysis file (.daf)
To use a custom template for analysis
Select a template or data analysis file (.ast, .daf)
OK
Cancel

2 To select the .cif files to merge, click **Add Files**.
The .cif file names appear in the list.
3 If you want to remove a file from the list, select it and then click **Remove File**.
4 Type a unique name for the output files.
5 Select a template..
6 Click **OK**.
7 The merged files are created and the new .daf file is loaded with a population created from each file.

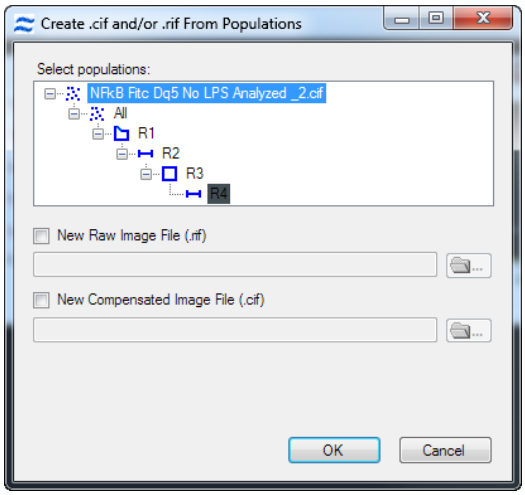
Creating New Data Files

Creating new data files from populations

To further analyze a population or merge it with other data when working in a .daf, you can save it to a new data file. This course of action is useful if your data file contains a large number of objects that are not pertinent to your experiment. Decreasing the data file size results in better performance by the IDEAS application, as described in Creating Regions on Graphs. Note that you cannot create a new .cif or .rif when multiple data files are open. This option is not available for basic FlowSight files without the Quantitative Imaging (QI) upgrade.

To create data files from populations

1 On the **Tools** menu, click **Create Data File from Populations**.
The **Create .cif and/or .rif From Populations** window appears.



2 In the **Select populations** list, select the populations that you want to include in the new data file(s). Ctrl click to select multiple populations.

3 To create a .rif file, select the **New Raw Image File (.rif)** check box, the population name is used as a default. You may enter a new name.

4 To create a .cif file, select the **New Compensated Image File (.cif)** check box, the population name is used as a default. You may enter a new name.

5 Click **OK**.
If you created a new .cif file, you can choose to load it. When loading the .cif file, the application will prompt you for the template.

Viewing Sample Information

All of the information associated with an IDEAS file, such as the collection information, camera settings and corrections, is saved within IDEAS and can be viewed in the Sample Information window.

To open the Sample Information window

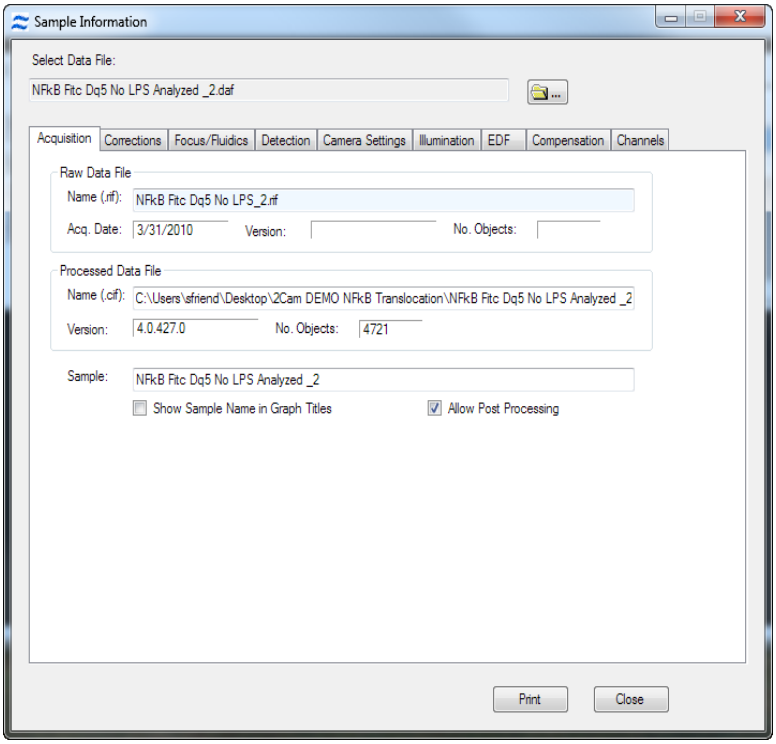
1 Go to **Tools** > **Sample Information** to open the window.

Information for the open data file will be loaded. You can browse for a data file by clicking on the folder. You can open the Sample Information Window for any of three file types: .rif, .cif, or .daf.

2 Select a Tab to see the information for each heading.

3 Click Print to print a report of all of the sample information.

Tip: You may click on the folder and browse for a file to view the sample information for any file without loading the file.



Acquisition tab: File names, software version numbers, date acquired, number of objects, sample name.

Corrections tab: Camera background, alignment offsets (from ASSIST).

Focus/Fluidics tab: Core information and sample volume.

Detection tab:**IS100 and ImageStreamX only**. Cell classifier settings during acquisition.

Population tab: **FlowSight and lageStreamX MKII only**. Lists the populations and number acquired.

Camera Settings tab: Bin mode, magnification and sensitivity settings.

Illumination tab: Brightfield and laser information.

EDF tab: View kernels used for deconvolution of EDF imagery.

Compensation tab: View the compensation matrix.

Channels tab: Lists channels collected.

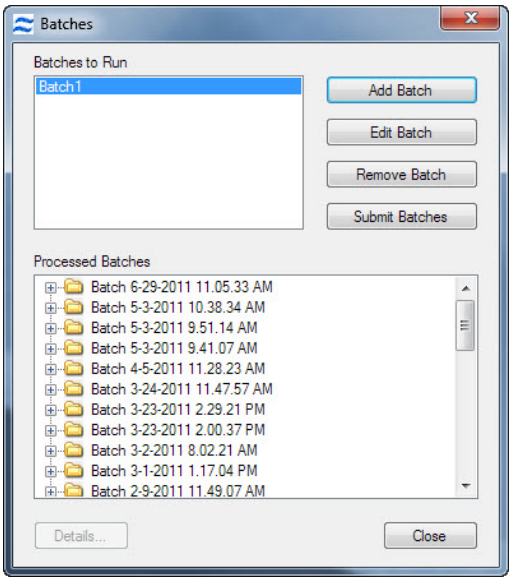
Batch Processing

Batch processing allows you to automatically analyze a group of files with one template when a compensation matrix has already been generated for the experiment.

To perform batch processing

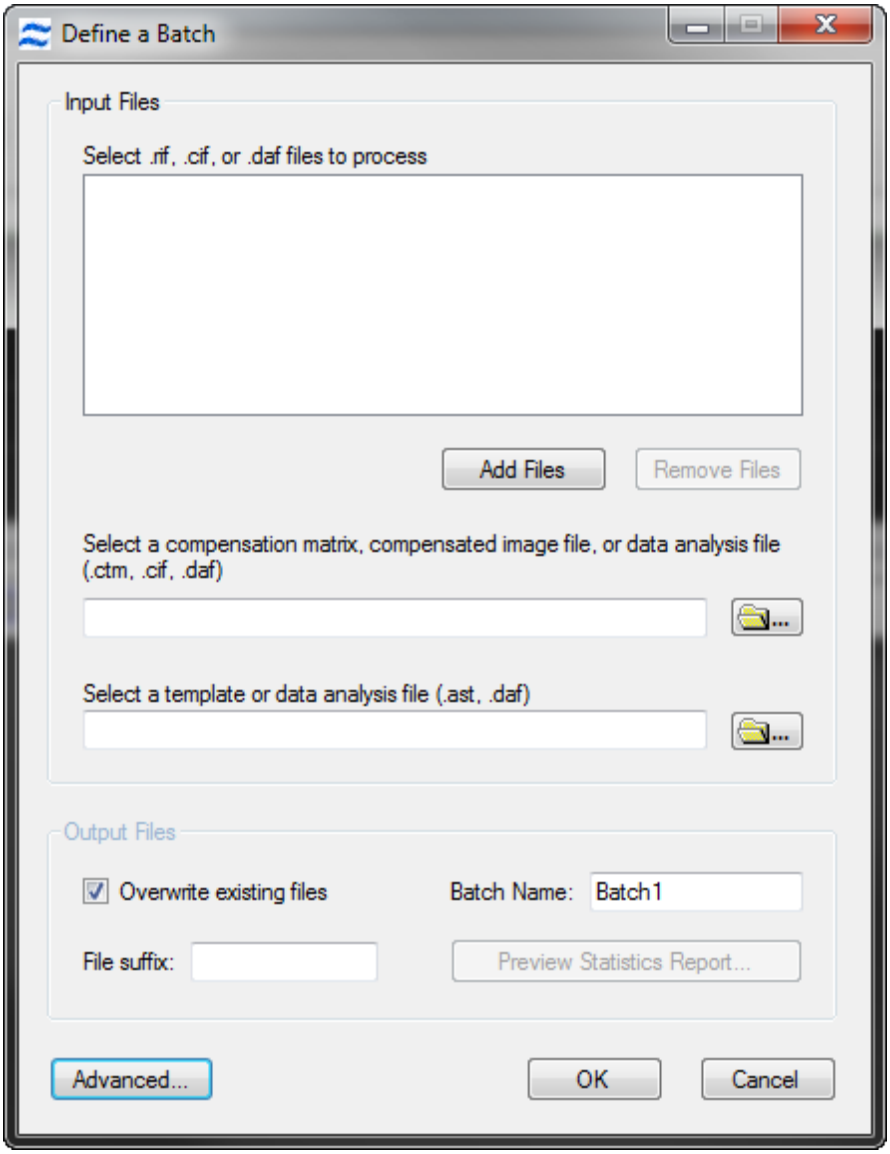
1 On the **Tools** menu, select **Batch Data Files**.

The **Batches** window appears. It lists a record of all batches you have processed.



2 Click **Add Batch**.

The **Define a Batch** window appears.



3 To select the files for the batch, click **Add Files**. Navigate to the files and select by clicking on the file. Select multiple files to add by holding down the Ctrl key while selecting the files.

- To remove files from the **Files to Process** list, click **Remove Files**.

4 Select a compensation matrix from a file (.ctm, .cif, or .daf).

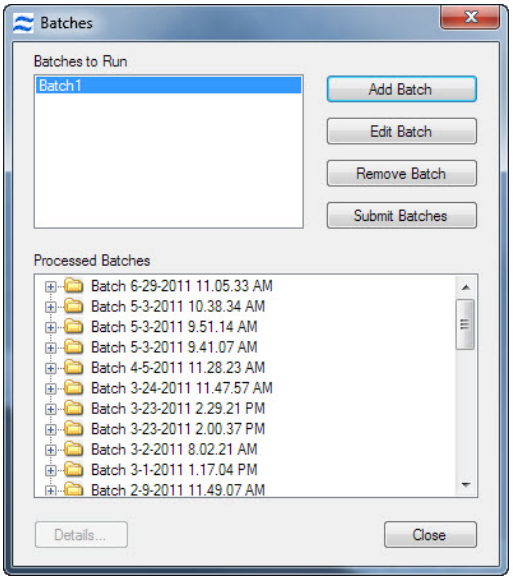
5 Select a template file (.ast or .daf). Leave blank to use the Default template.

6 Set the output files parameters.

7 If the template contains a Statistics Report template click on the **Preview Statistics Report** button. Order the files as you wish them to be reported by selecting a file with a left-click, then right-click the desired position and select 'move here'. See Reporting Statistics for more information.

8 Click **OK**.

The **Define a Batch** window closes. The batch appears in the **Batches** window.

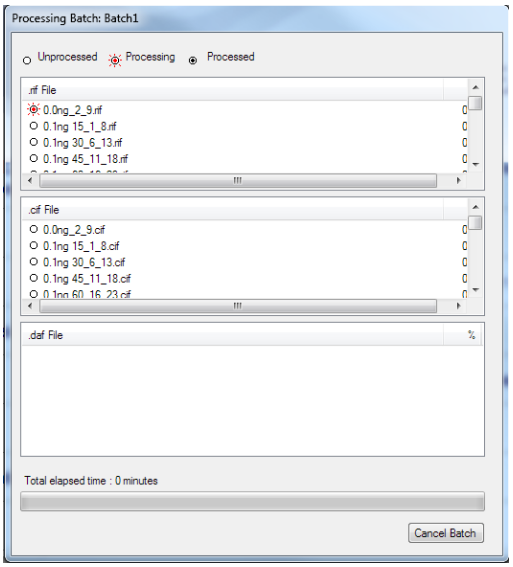


9 The Batches window offers the following options:

- **Add Batch:** If you want to create another batch to add to the list.
- **Remove Batch**: If you want to remove a batch from the Batches to Run list.
- **Edit Batch**: If you want to edit a batch in the Batches to Run list.

10 When you are satisfied with the Batches to Run list, click **Submit Batches**.

The files to process are listed and the progress is displayed in the Processing Batch window. Once you have started processing batches, it may use up a fair amount of your computer's processing power.



Tip: To cancel the batch processing at any time, click **Cancel Batch**. The IDEAS application will confirm cancellation and complete the file it is working on.

When the batch processing is complete, the IDEAS application saves the .rif, .cif, and .daf files in the batch results directory. In the Batches window, a list of

processed batches appears in the Processed Batches list. If a batch did not successfully complete, it will appear in red.

Tip: To display the error that occurred during processing, double-click the batch.

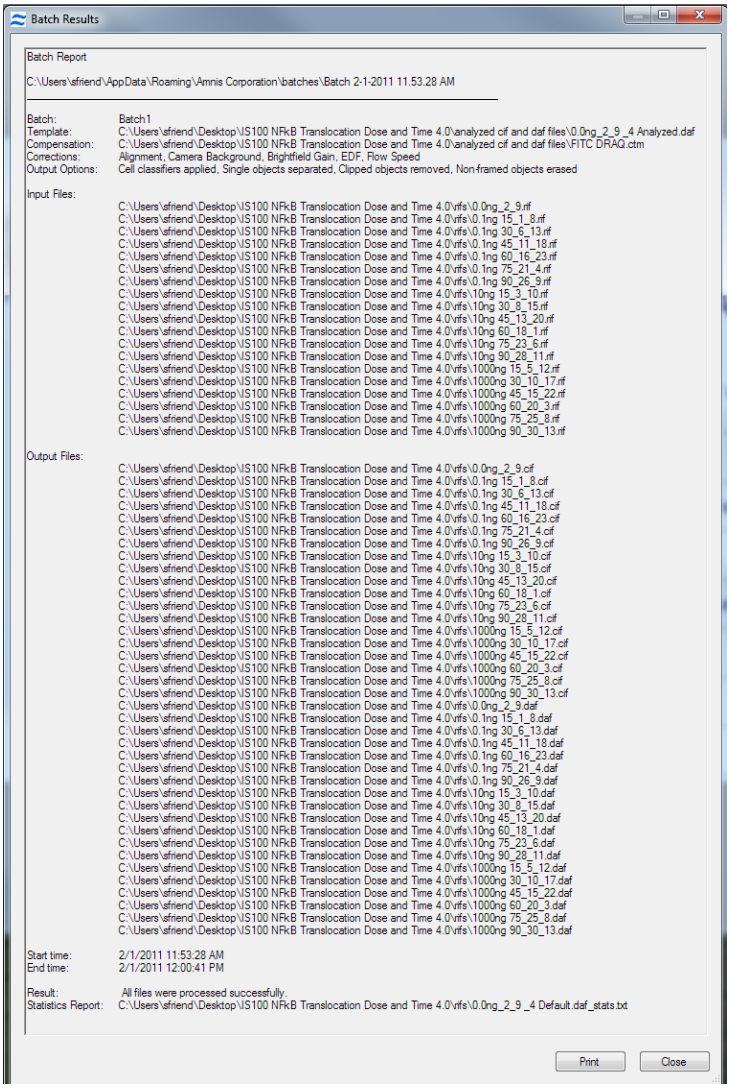
11 If you want a batch report, double-click the batch in the Processed Batches list of the Batches window.

The Batch Results window appears.

12 In the Batch Results window, click **Print**.

13 In the Batch Results window, click **Close**.

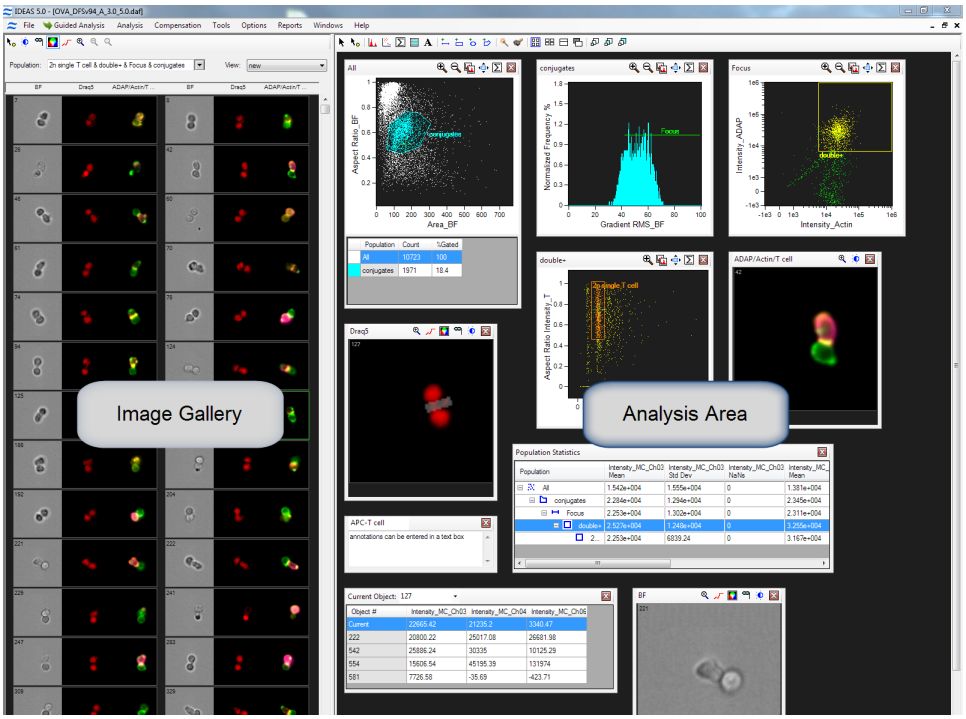
14 In the Batches window, click **Close**.



Overview of the Data Analysis Tools

The IDEAS application provides a powerful tool set that allows you to explore and analyze data. The rich feature set lets you create hundreds of your own features to differentiate objects and statistically quantify your results.

As shown in the following figure, the application window is divided into two panels–the Image Gallery and Analysis Area–which each provide the corresponding tools that you can use for data analysis. The layout can be changed to side by side or top and bottom with re-sizable panels.



You can create populations of objects by tagging hand-selected images, drawing regions on graphs, and using Boolean logic to combine existing populations. After you have created a population, you can view it in the Image Gallery or plot it on a graph. You can view the statistics for populations or objects in tables placed in the analysis area.

Graphs show data plotted with one or two feature values, and tools are provided that allow you to draw regions for the purpose of generating new populations. You can show any population on a plot.

Every image is linked to the feature data. Selecting an individual data point in a graph allows you to view it in the Image Gallery or look at its feature values in the Statistics Area. Any object that is selected in the Image Gallery is also shown on the plots in the Analysis Area.

Overview of the Image GalleryUsing the Image Gallery

This section contains the following subsections, which describe how to view populations of objects in various ways, view masks, customize the Image Gallery display, and hand-select objects for a population:

Overview of the Image GalleryUsing the Image Gallery

Setting the Image Gallery Properties

Working with Individual Images

Creating Tagged Populations

Overview of the Image Gallery

The Image Gallery displays the imagery and masks of any population of objects.

A toolbar is provided in the upper-left corner of the panel, as shown in the following figure. The Image Gallery also makes different viewing modes available for the imagery. The default template contains the viewing modes which allows you to view all channel images in grayscale or color, or each channel image individually.

Tip: You can build custom viewing modes as shown in this example. For more information, see Setting the Image Gallery Properties.

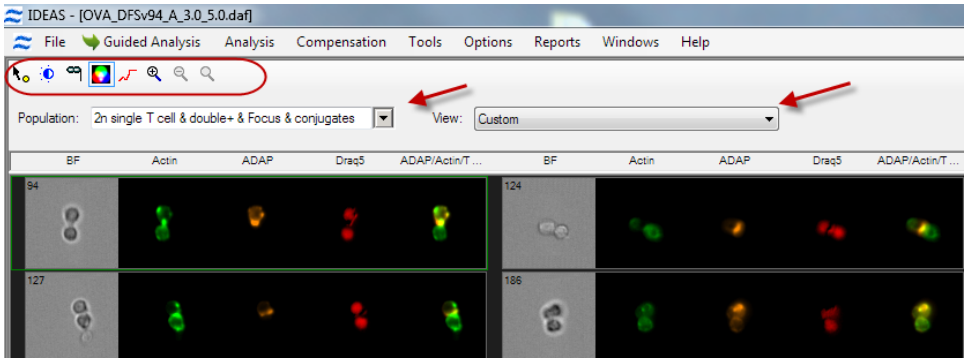


Image Gallery Tools

Table 1: Image Gallery Tools

Tool	Description
Tagging Mode Tool	Allows you to create a population of hand-picked objects. See: Creating Tagged Populations.
Image Gallery Properties Tool	Provides custom display features. See: Setting the Image Gallery Properties.
Show/Hide Mask Tool	Displays masks on the imagery. See: Overview of the Image GalleryUsing the Image Gallery.

Tool	Description
Show/Hide Color Tool	Sets the Image Gallery color. See: Overview of the Image GalleryUsing the Image Gallery.
Show/Hide Saturated Pixels Tool	Click on the tool and it will show any saturated pixels will turn red. See: Overview of the Image GalleryUsing the Image Gallery.
Zoom Tools	Zoom in or out and reset zoom on the image gallery. See: Overview of the Image GalleryUsing the Image Gallery

To view the imagery for a population

1 In the **Population** drop down menu of the Image Gallery, click the population that you want. (The list includes all the populations as well as the currently selected bin from a histogram.) To create a population, refer to Creating Tagged Populations.

2 To select an individual image, click on it. A thin, green frame indicates the selected object.
   - The object's feature values are displayed in a table if an object is selected and a table is added to the Analysis Area.
   - The selected object is identified in each scatter plot graph with a green cross.
   - The image can be placed in the Analysis area by **right click>Add Image to Analysis Area**.

     Tip: Conversely in any scatter plot in the analysis area, clicking a graphical point causes the Image Gallery to highlight and display the corresponding object.

     Note that the objects are presented in the Image Gallery in the order of acquisition and are not necessarily near each other in a scatter plot.

To change the viewing mode

- In the **View** drop down menu of the Image Gallery, select a specific view. The imagery display changes according to the new view. To make a new view use the Image Properties tool.

To show or hide masks

- Click the **Show/Hide Masks** toolbar button to toggle between showing and hiding the selected masks for all images in the Image Gallery .

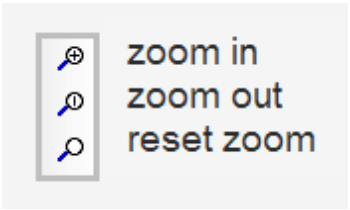
  The mask is shown as a transparent layer over each image. The mask displayed is selected in the Image Gallery Properties View tab. The color of the overlayed mask can be changed in the Applications Defaults under the Options menu.

Tip: To hide the mask for a specific channel only, set the individual channel mask to None in the view tab of the Image Properties dialog.

To show or hide color

- Click the **Show/Hide Color** toolbar button to toggle between showing and hiding the colors for all images in the Image Gallery. See Setting the Image Gallery Properties for more information.

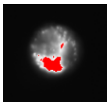
To zoom on the image gallery

- Click the **Zoom In** toolbar button to view the images in the gallery closer and the **Zoom Out** or **Reset Zoom** to reverse the zoom.
- 

To show saturation

- Click the **Show/Hide Saturated Pixels** toolbar button.

Saturated pixels in images, if any, appear in red.



Setting the Image Gallery Properties

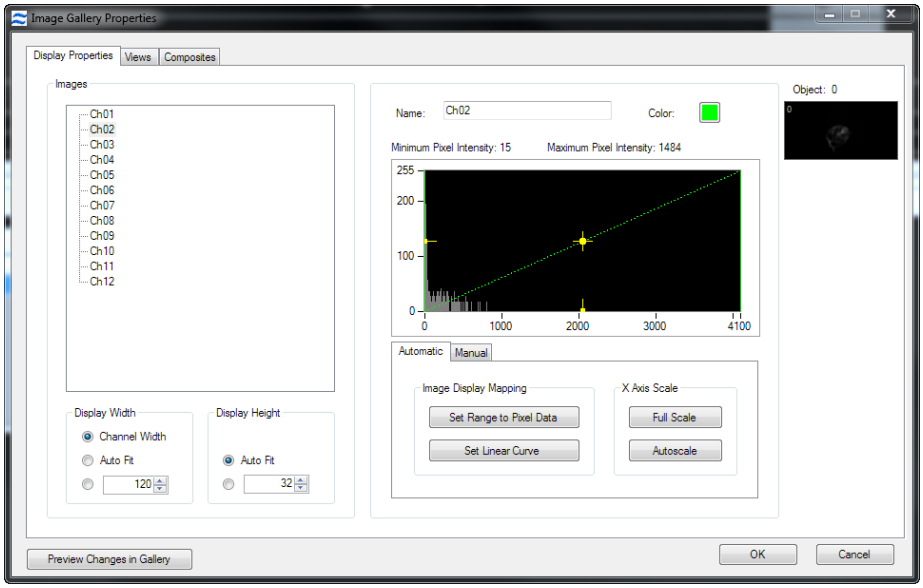
When a new data file opens in the default template, you might find it difficult to clearly see cell morphology because the Image Gallery display properties have not yet been properly adjusted for the data set.
To optimize the display you may use the wizard Display Properties Wizard to set the pixel intensity mapping to the display range. Manual adjustment and other settings are described below.
Clicking the **Image Gallery Properties** toolbar button opens the Image Gallery Properties window, which contains the following tabs:

- **Display Properties**–Allows you to define the name, color, and display intensity mapping for each image. Allows adjustment of the image size for the image gallery.
- **Views**–Allows you to customize the views for the Image Gallery.
- **Composites**–Allows you to create composites and adjust the amount of color from a channel that is included in a composite image.

To customize the Image Gallery display properties

1 Click the **Image Gallery Properties** toolbar button to begin.

The **Image Gallery Properties** window appears with the **Display Properties** tab displayed.



To change the size of the panels in the image gallery

1 Display Width and Display Height can be specified or changed to Auto Fit in the lower left section of this window.

To change the name or color for each image

1 Select an image in the list of images on the **Display Properties** tab of the **Image Gallery Properties** window.

2 On the right side of the window you can type a new, unique name for the selected image. Note that each image is provided with a default name and the image names appear near the top of the Image Gallery.

3 Click the colored square for the selected image.

4 Click the color that you want in the color palette.

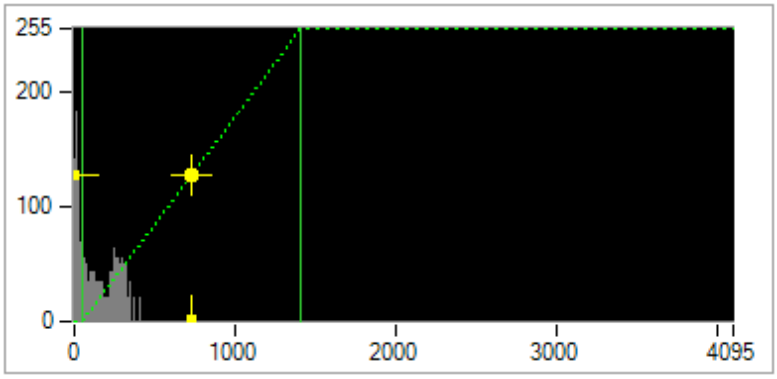
5 Click **OK** to close the palette.

Tip: The grayscale image in each channel is assigned a default color for image display in the gallery. Setting the color to white is equivalent to using the original grayscale image. The colors are also used to build composite images.

To fine-tune the image display intensity for an image

1 On the **Display Properties** tab of the **Image Gallery Properties** window, select an image by clicking the image name in the list. The graph for the currently selected image is shown in the window and updates as the changes are made. Select and image in the image gallery that has intensities for the image channel you are adjusting.

Note: You will adjust the **Display Intensity** settings on the graph (the Y Axis), the value of the display to (the X axis), the range of pixel intensities. The range of pixel intensities will depend on the instrument and the collection mode set during acquisition. The display range is 0-255; the range of intensities from the camera is 0-4095 for the ImageStream^X or 0-32,767 for EDF mode collection. The IS100 first generation instrument has a 10 bit camera and therefore the range of pixel intensities is 0-1023. The limits of the graph enable you to use the full dynamic range of the display to map the pixel intensities of the image.



At each intensity on the X Axis of the graph, the gray histogram shows the number of pixels in the image. This histogram provides you with a general sense of the range of pixel intensities in the image. The dotted green line maps the pixel intensities to the display intensities, which are in the 0-255 range.

Manual setting is done by Click-dragging the vertical green line on the left side (crossing the X Axis at 0) allows you to set the display pixel intensity to 0 for all intensities that appear to the left of that line. Doing so removes background noise from the image.

Click-dragging the vertical green line on the right side allows you to set the display pixel intensity to 255 for all intensities that appear to the right of that line.

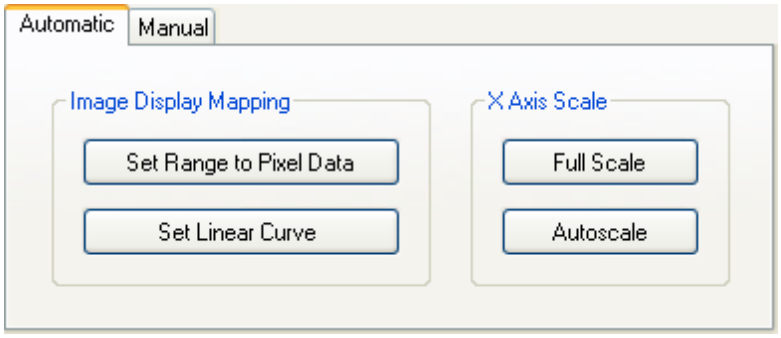
2 From the Image Gallery window, select the object to use for setting the mapping. It appears in the Image Gallery Properties window.

Tip: You might need to select different objects for different channels because an object might not fluoresce in all channels.

3 To adjust the pixel mapping for display, click-drag the vertical green line by clicking near it (but not near the yellow cross).

Tip: For fluorescence channels, set the vertical green line that appears on the left side to the right of the large peak of background pixel intensities as shown above and set the right vertical green line to the right of the brightest pixel intensities. Click 'Set Linear Curve' to make the transformation linear. For the brightfield channel, set the vertical lines to about 50 counts to the right and left of the histogram to produce an image with crisp brightfield contrast.

- To change the mapping curve to be logarithmic or exponential, click-drag the yellow cross.
- To restore the mapping to a linear curve, Click **Set Linear Curve**.
- To see the full scale for the X Axis Click **Full Scale**.
- To set the display mapping of the X Axis to the lowest and highest values for a selected object, Click **Set Range to Pixel Data**.
- To set the scale of the X Axis to the range of the vertical green lines or of all the pixel intensities for the selected object–whichever is larger–Click **Autoscale**.
- You may enter values manually by selecting the **Manual** tab.



4 If you want to preview the changes in the Image Gallery, click **Preview Changes in Gallery**.

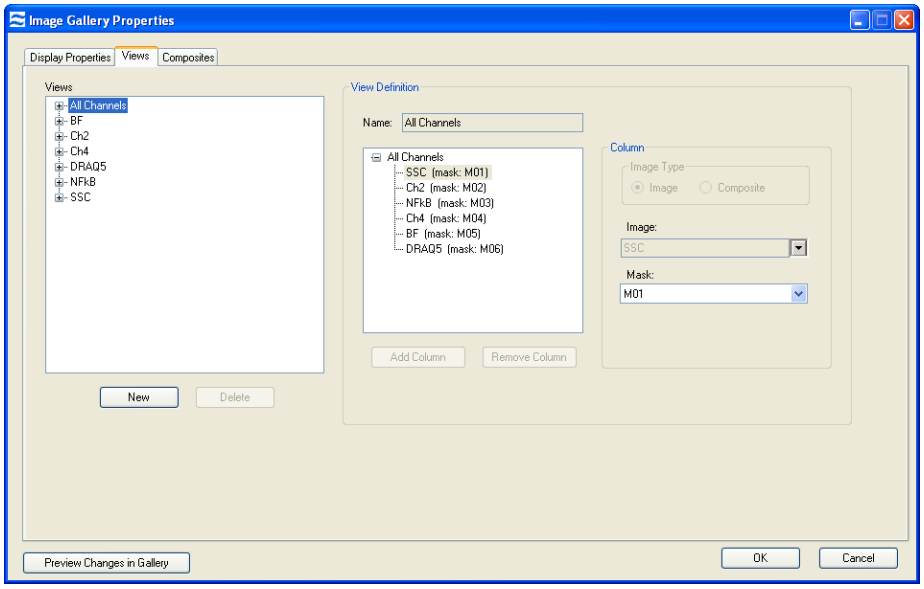
5 Continue customizing the Image Gallery display properties with another procedure in this section, or click **OK** to finish and save changes or **Cancel** to finish and discard changes.

To customize the Image Gallery views images and masks

1 Within the **Image Gallery Properties** window, click the **Views** tab.

Note: The Image Gallery view can be customized to view any combination of channel images or composites. The default view **All Channels** is a view that displays all image channels that were included during acquisition of the file with their associated default masks. This mask may be changed for the default view however, the images in this view cannot be changed.

The list of existing views is shown on the left.



2 To create a new view, Click **New**. The new view is automatically added to the list on the left.

3 In the right section of the window, type in a name for the view.

4 Click **Add Column**.

5 Define the column by selecting an image and a mask or a composite from the dropdown menu.

6 Repeat the previous 2 steps until finished adding columns to the view. A column will be added under the column currently selected. To insert a column click on the image above insertion point.

7 Columns may be removed by clicking on **Remove Column**.

8 A view may be edited at any time by selecting the view and following the same procedures.

9 If you want to delete a view, click the view to select it, and then click **Delete**.

A confirmation window appears.

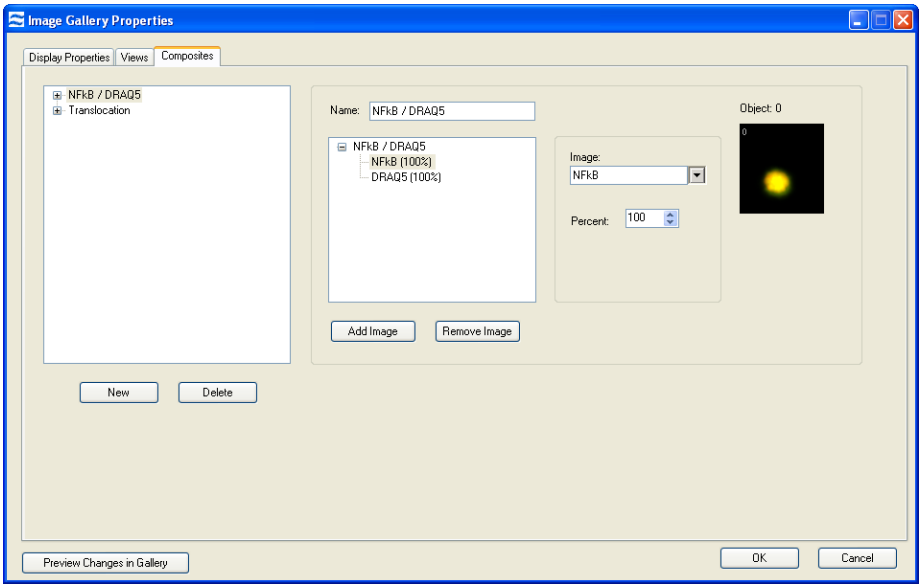
10 If you want to preview any new changes in the Image Gallery, return to the Image Gallery and choose your new view in the **View** drop down menu. Then return to the **Image Gallery Properties** window and click **Preview Changes in Gallery**.

11 Continue customizing the Image Gallery display properties with another procedure in this section, or click **OK** to finish and save changes or **Cancel** to finish and discard changes.

To create a composite

1 Within the **Image Gallery Properties** window, click the **Composites** tab.

The list of existing composites is shown on the left.



2 In the right section of the window, type a name for the composite or leave blank to allow the name to be built automatically from the image names added to the composite.

3 Click **Add Image**. The selected image appears in the **Object** box. Change the **Percent** if desired. The percent specifies the percentage of of the image to include in the composite.

Tip: As you make the changes, the image in the **Object** box updates accordingly. If you want to preview any new changes in the Image Gallery, return to the Image Gallery and select the **View** drop down menu to your new view. Then return to the **Image Gallery Properties** window and click **Preview Changes in Gallery**.

4 Continue to add images as desired.

5 To remove and image from the composite, Click **Remove Image**.

6 The composite is automatically added to the list on the left.

7 A composite can be removed from the list by clicking **Delete**.

8 Continue customizing the Image Gallery display properties with another procedure in this section, or click **OK** to finish and save changes or **Cancel** to finish and discard changes.

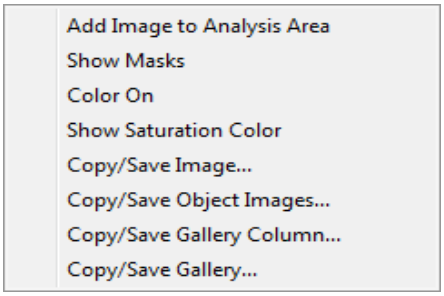
Working with Individual Images

You can work with individual images in the Image Gallery. You can zoom in or out on the images. You can add a larger version of an image to the Analysis Area for further analysis, show or hide masks for a single image in the Image Gallery, and copy one or more images to the Clipboard.

To manipulate individual images

1 In the Image Gallery, right-click an image that you are interested in.

A menu appears.

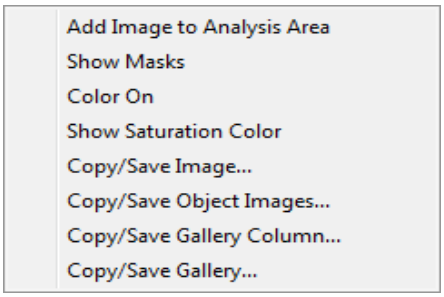


- To place the image in the Analysis Area, click **Add Image to Analysis Area**. (For more information, see Analyzing Individual Images.)
- To show or hide the masks for the object image, click **Show Masks** or **Hide Masks**, respectively. (One or the other will appear depending on the current state.)
- To turn the colors on or off for the object image, click **Color On** or **Color Off**, respectively.(One or the other will appear depending on the current state.)
- To show or hide the saturation color for the object image, click **Show or Hide Saturation Color** respectively.(One or the other will appear depending on the current state.)

To copy or save images for use in reports

1 In the Image Gallery, right-click an image that you are interested in.

A menu appears.



- To copy or save the single channel image to the Clipboard, choose **Copy/Save Image**
- To copy or save all of the channel images of one object, choose **Copy/Save Object Images**.
- To copy or save the single channel image for all of the displayed images to the Clipboard, choose **Copy/Save Gallery Column**.
- To copy or save all the visible images in the Image Gallery choose **Copy/Save Gallery**.

Overview of the Mask Manager

This section contains the following subsections, which describe how to create, edit, and delete a mask:

Creating New Masks with the Mask Manager

Overview of the Mask Manager

A mask defines a specific area of an image to use for feature-value calculations. The IDEAS application contains a Mask Manager for viewing existing masks and creating new ones.This option is not available for basic FlowSight files without the Quantitative Imaging (QI) upgrade.

When the IDEAS application loads a .rif file, the application creates a segmentation mask for each channel image and stores the mask along with the image in the .cif file. The masks, labeled M01 through M12, contain pixels that are detected as brighter than the background. In addition, the application generates a Combined Mask, named MC and a Not Combined Mask, Not MC for each object. A combined mask consists of the union of the masks of all the channels of the object. A Not Combined Mask is all of the pixels with no intensities above background.

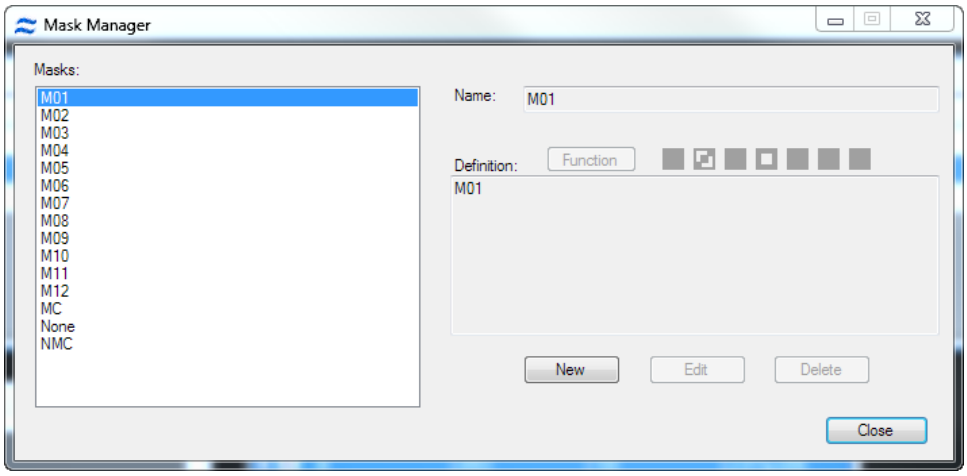
You might need to adjust the masks or create new ones that include only a specific area of a cell, such as the nucleus. You can combine masks by using Boolean logic, or you can adjust them by applying functions.

Creating New Masks with the Mask Manager

There are two ways to work with new masks in the Mask Manager. First, masks can be created by using functions, which allows you to choose an input mask and, if needed, adjust the channel and scalar input. Alternatively, masks can be created by combining masks through Boolean logic. This option is not available for basic FlowSight files without the Quantitative Imaging (QI) upgrade.

To create a new mask using Functions

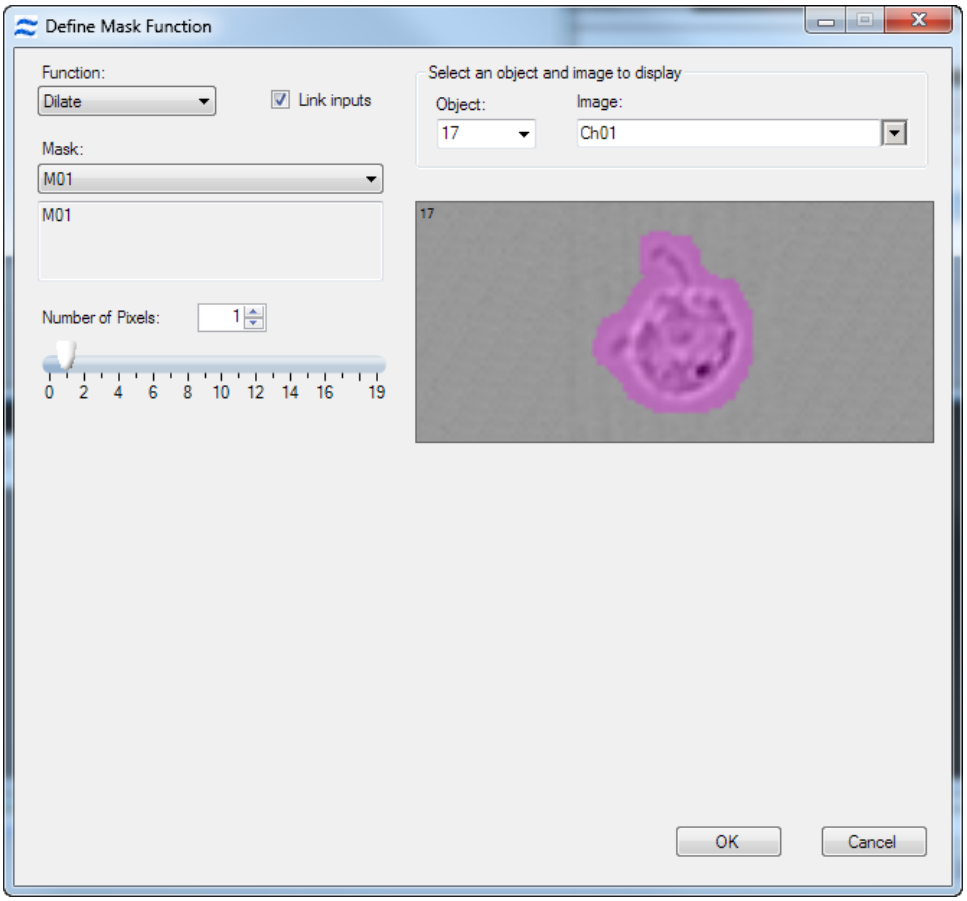
1 Select **Analysis** > **Masks**.
The Mask Manager opens with a list of existing masks on the left.
2 Click **New**.
The right side of the window is enabled to define a new mask.



3 Click **Function**.

The Define Mask Function window appears with 15 available masks to use. SeeAbout Masks for mask definitions.

- **Dilate**
- **Erode**
- **Fill**
- **Inspire**
- **Intensity**
- **Interface**
- **Morphology**
- **Object**
- **Peak**
- **Range**
- **Skeleton**
- **Spot**
- **System**
- **Threshold**
- **Valley**



4 Select a function and choose the input mask(s), channel and scalar parameters as needed. The right side of the window adjusts the display and view of the channel image.
   - To view a different object in the file, select it in the **Object** list or type it's number.
   - To view a different image for the object, select it from the list.
5 The Link inputs checkbox is checked by default. To modify a mask with different inputs uncheck this box.
6 Click **OK.**
7 The new function is added to the mask definition.
8 Click **OK**.

   The new mask name will appear in the list of Masks on the left side.

To create a new combined mask

1 Select **Analysis** > **Masks**.
2 Click **New**.
3 Use the **Masks** list on the left and the **Definition** toolbar to build a new mask using the definitions of existing masks with Boolean logic explained in the table below.

Table 1: Mask Tasks and Toolbar

Task	Toolbar
Add a mask to the definition.	Double-click the feature in the **Masks** list. Or, single click the feature in the **Masks** list and click the leftmost down-arrow button on the toolbar.
Combine two masks.	Use the Boolean AND or OR operator. Use the AND operator to include only the pixels that are in both of the original masks. Use the OR operator to include the pixels that are in either one of the original masks.
Select all pixels that are not in the original mask.	Use the Boolean NOT operator. The NOT operator specifies which mask will not be used.
Affect the order of operations.	Use the parentheses toolbar buttons.
Remove an item from the end of the definition.	Click the left-arrow button on the toolbar.

4 Add masks and Boolean logic to the definition as needed.

5 Click **OK** to add the definition to the Masks list.

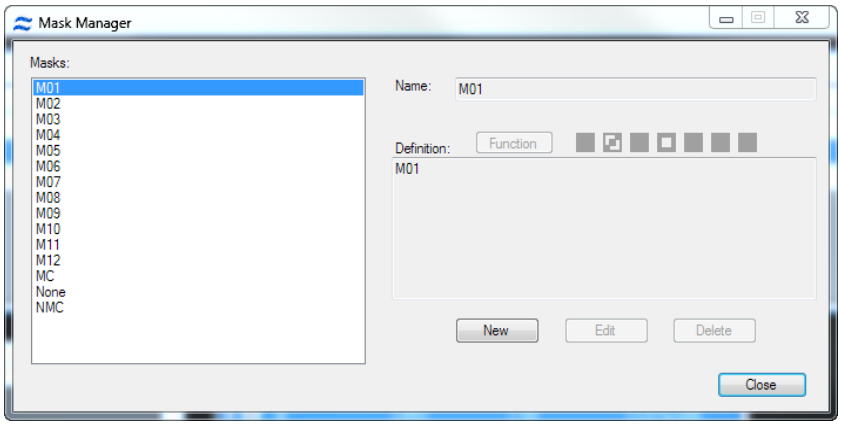
6 Click **Close**.

Viewing and Editing a Mask

To view a mask definition

1 Select **Analysis** > **Masks**.

The **Mask Manager** window appears.



2 Click a mask in the **Masks** list to view the definition in the **Definition** area.

3 Click **Close**.

To edit a mask function

1 In the Mask Manager window, select the mask that contains the function you want to edit.
2 Click **Edit**.
3 Remove the definition for the combined mask using the back arrow tool as needed.

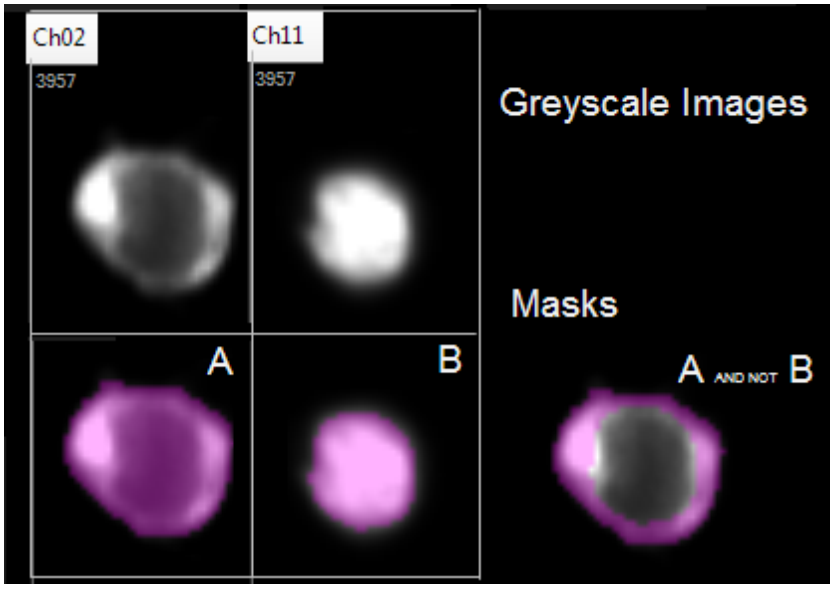
4 Or click the Function button on the toolbar for a function mask. The **Define Mask Function** window appears.

5 Click **OK** when finished.

Example of Creating a Mask

Here is an example of creating a mask of the cytoplasm.

In this example, cells were stained with a green intracellular marker (in Channel 2) and a red nuclear dye (in Channel 11). You can generate a cytoplasm-specific mask by first refining the intracellular and nuclear masks and then removing the nuclear mask pixels from the intracellular mask.



1 Observe the default masks in the Image Gallery. Since the default masks are designed to capture all the light in an image, they tend to include light that exists beyond the perceived boundaries of the images. In this case, both the intra-cellular and nuclear masks need to be refined. Start by creating morphology masks for both channel images because the Morphology mask is designed to conform to the shape of the image. Note that the Object mask function may also be used in place of the Morphology mask function.
2 Select **Analysis** > **Masks**.
3 Click **New**.

4 Click on the Function toolbar button to adjust the mask that will define the whole cell. The **Define Mask Function** window appears.

5 Select Morphology in the Function list.

6 Select a starting Mask.

7 Select Channel 2 (intracellular marker) on the left side of the window.

8 Click **OK**.

9 Click **Set Default Name** or, enter a new mask name.

10 Click **OK** to add this mask to the list.

11 To make the Morphology(Nuclear) mask, repeat steps 3-10 using Channel 11.

12 Click **Close**.

13 To view the resulting morphology masks, open the Image Display Properties window and, if necessary, select the new mask(s) for the channel.

(Icon for Image Display Properties)

14 Next, you will subtract the nuclear morphology mask from the intracellular mask. In the Mask Manager window, click **New**.

15 Double-click the Morphology(Intracellular) mask in the list.

16 Click the AND button on the toolbar.

17 Click the NOT button on the toolbar.

18 Double-click Morphology(Nuclear) mask in the list.

19 Enter a new mask name.

20 Click **OK** to add this mask to the list.

21 Click **Close**.

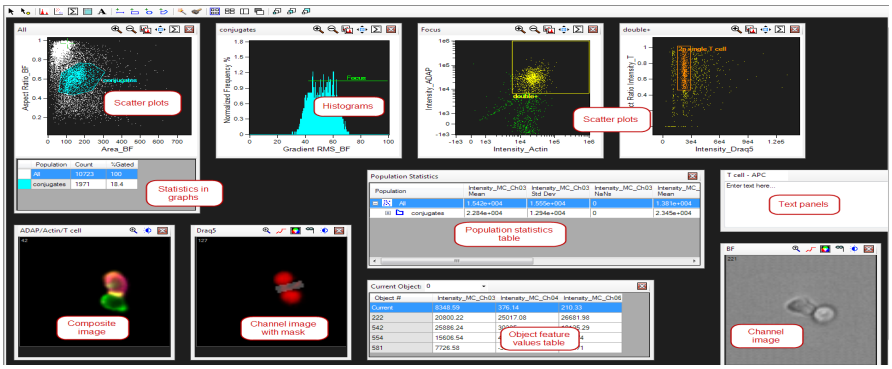
22 To view the resulting mask on a Channel 3 image, open the Image Display Properties window,and select the new mask for the channel in the view you are using.

Overview of the Analysis Area

The Analysis Area provides display space for individual images, plots of cellular feature values, tables of population statistics, tables of object feature values, and text annotations. You can select different layouts for the IDEAS window and placement of the analysis area and expand the Analysis Area by dragging it's boundaries.

The graphs are created into panels of a default size and can be re-sized by dragging a corner or using the size tool. The position of the panels is automatically adjusted to fit in the available display space. A vertical scroll bar appears when the number of panels exceeds the space available on the window. The panels can be re-tiled using the arrange analysis area tool.

As illustrated by the following figure, the Analysis Area can contain several types of panels: histogram, histogram overlay, scatter plot, tables of population statistics or object feature values, channel image, composite image, and text. Each panel will contain its own toolbar and context menu. To move a panel click on the name at the top of the graph and drag it to a new location. A graph may be selected and then a right click in a blank space in the work area allows you to choose paste in the new location.

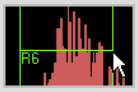


A toolbar is visible at the top of the Analysis Area. The following table describes the function for each tool.

Analysis Area Tools

Table 1: Analysis Area Tools

Tool	Description
Pointer Tool	Provides the normal mode of interaction with the graphs. Clicking a point on a scatter-plot graph causes the IDEAS application to display the corresponding image in the Image Gallery (if the population that is currently displayed in the Image Gallery contains that point). Click the bin in a histogram to select the bin. In the Image Gallery, you can view images of cells in the bin by choosing the Selected Bin population.

Tool	Description
	Click Pointer Tool while drawing a region on a graph to cancel the creation of a region.
Tagging Tool	Allows you to create a population of hand-picked objects. For more information, see Creating Tagged Populations.
New Histogram Tool	Creates a new histogram.
New Scatter Plot Tool	Creates a new scatter plot. Refer to
Populations Statistics table	Creates a table to display population statistics.
Object Feature Values table	Creates a table to display selected object feature values.
Text Tool	Allows user to add text notes to the Analysis Area. Refer to Adding Text to the Analysis Area.
Line Region Tool	Draws a horizontal line on a histogram to define a region. 
Rectangle Region Tool	Draws a rectangular region on a scatter plot.
Oval Region Tool	Draws an oval region on a scatter plot.
Polygon Region Tool	Draws a polygon region on a scatter plot graph. Each click starts a new segment in the polygon until the entire image is double-clicked to complete the region.
Wizards Tool	Short-cut to using Wizards for guided analysis.
Building Blocks Tool	Short-cut to using Building Blocks for guided analysis.
Select All Tool	Selects all panels in the analysis area.
Tile Graphs Tool	Tiles graphs in the analysis area after changing the size of the analysis area to fit all graphs to the new space.
Layout Tools	Switches the layout of the image gallery and analysis area.
Graph Bkgd Tool	Changes the background of the graphs to black or white.
Graph sizing Tools	Changes the size of selected graphs to small, medium or large.

Creating Graphs

You can add two types of graphs to the Analysis Area:

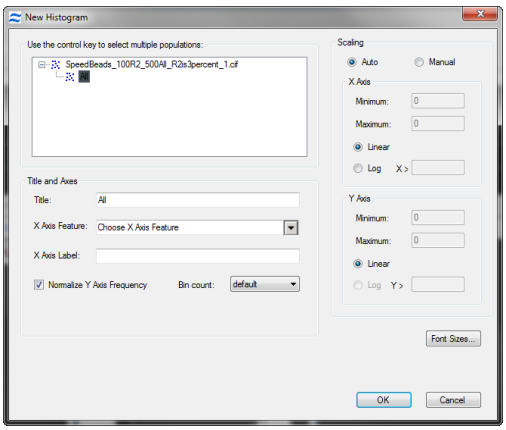
- Histogram–Graphs a single feature.
- Scatter Plot–Graphs two features.

Note that building blocks are available that will help you to create graphs for finding single, focused, fluorescent positive events or a size versus scatter plot. See Building Blocks.

To create a graph without using a building block

1 Click the New Histogram or New Scatter Plot toolbar button.

The New Histogram or New Scatterplot window appears, respectively.



2 Select the one or more populations to graph by clicking them. To select more than one population, use the Ctrl key.

The title defaults to the selected population. You can edit the title.

3 In the **X Axis Feature** drop down menu, select the feature that you want to graph on the X Axis.

4 If you want to change the label for the X axis, edit the text in the **X Axis Label** field.

The label defaults to the name of the selected feature.

5 If you are creating a scatter plot, select a feature and a label for the Y Axis. If you are creating a histogram you can choose to normalize the Y axis frequency and change the bin count.

6 The default font sizes are used, you may change them by clicking **Font Sizes.**

7 Assign colors by Population (default) for dot plots or by Density for density plots.

8 Set the scaling for each axis of the graph. (The default is **Auto**, which allows the application to automatically scale the graph.)

9 To set minimum and maximum values for an axis, select **Manual**.

10 Select **Linear** or **Log** and enter **Maximum** and **Minimum** limits.

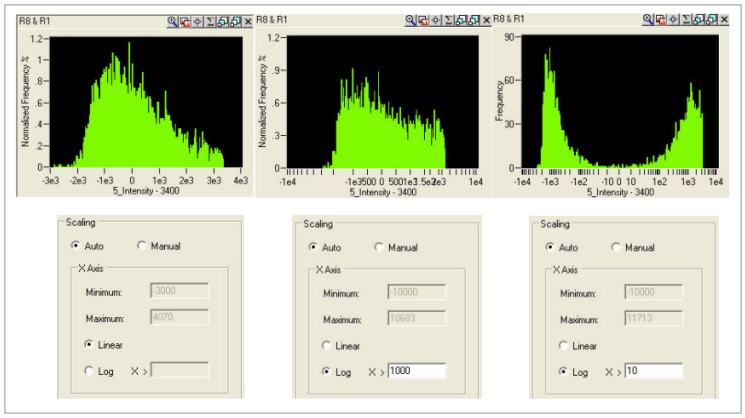
11 If you selected **Log**, enter the **X >** value.

Note: You can scale the X Axis of a graph or the Y Axis of a scatter plot in one of two modes: **Linear** or **Log**. The Linear mode is the default.

The **Log** mode allows you to logarithmically scale a section of the graph or scatter plot. Selecting this mode causes the IDEAS application to perform bi-exponential plotting. The > X value defines the linear portion of the graph as -X through X. The application plots the values outside of these limits on a log-

arithmic scale. You can plot negative values as well as positive ones on a logarithmic scale by adjusting the limits.

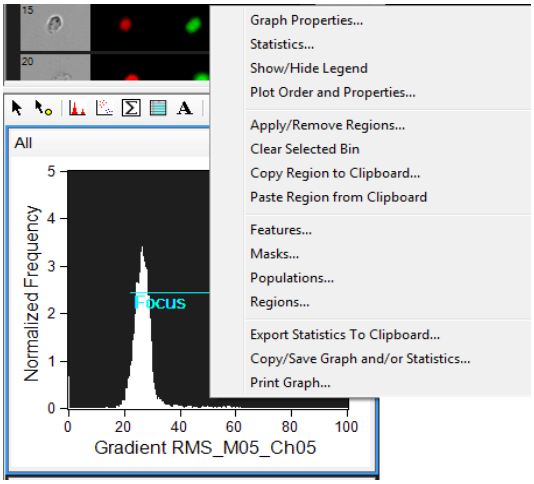
Take care not to split a population such that it appears to be two separate populations. This splitting is especially likely when negative values exist due to compensation or corrections on the imagery. The graph on the left side was plotted on a linear scale; the ones in the center and on the right side were plotted on logarithmic scales. The graph on the right side split the population because the change from a linear to a logarithmic scale occurred in the middle of the population. In general the >X value should be 1000 for 40 and 60X data and 100 for 20X data.



12 The font sizes can be changed for an individual graph.

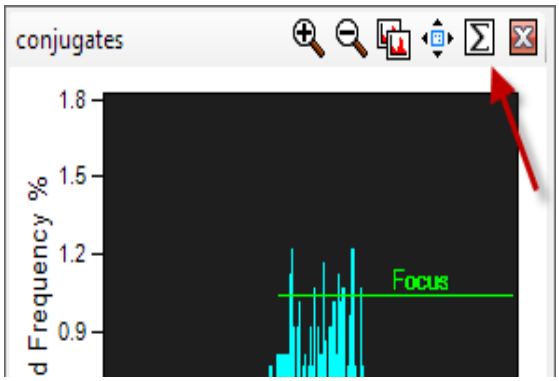
13 Click **OK**.

Tip: After you have created a graph, you can change its properties by right-clicking the graph and selecting **Graph Properties**. The same window that you used to create the graph will reappear, and you can then make any changes that you want.



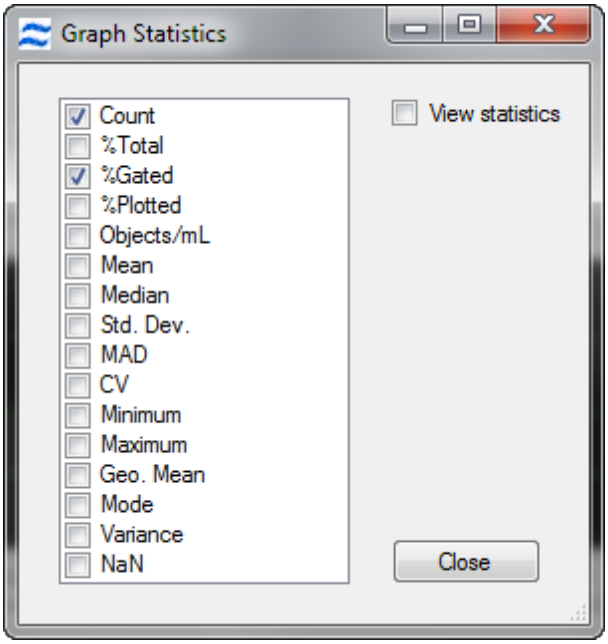
To show selected statistics for a graph

1 You can show and hide statistics by clicking the Statistics toolbar button in the panel that contains the graph.



2 Or, right-click anywhere on the graph and click **Statistics** on the graph context menu that appears.

The **Statistics** window appears.



3 Select the statistics that you want to display. Check the View statistics box and the box next to the statistic to be displayed for each population on the graph. The statistics that are supported are the Count, Percent Total, Percent Gated, Percent, Concentration (count/sample volume), Mean, Median, Standard Deviation, MAD (Median Average Deviation) RD -Mean, RD -Median, CV, Minimum, Maximum, Geometiric Mean, Mode, variance, and NaN (not a number).

4 When finished, click **Close**.

To show the legend for a graph

1 Right-click anywhere on the graph, and click **Show/Hide Legend** on the graph context menu that appears.

If the legend was hidden, it appears on the graph. If the legend was shown, it disappears from the display.

Note: The legend contains an entry for each population on the graph. If the graph is a scatter plot, the legend shows the population and its associated point style and color. If the graph is a histogram or overlay histogram, the legend shows the population name, associated color, and line type.

- To move the legend, click and drag it. (You cannot drag the legend past the boundary of the graph panel.)

To order the plots on a graph or change the fill and lines for a histogram

1 Right-click anywhere on the graph and choose **Plot Order and Properties** on the graph context menu that appears. The Display Properties dialog appears.

Moving a Graph

- With any graph in the Analysis Area, you can move it to another location by clicking in the center of the graph and dragging it. Alternatively, select the graph, right-click in the a blank space in the analysis area choose cut and then right-click where you would like to move the graph and choose past.

Creating Regions on Graphs

Regions may be drawn on graphs to create new populations, based on the physical location of objects on a graph, and to compute statistics. Tools for drawing regions are found on the Analysis Area toolbar. A line region may be drawn only on a histogram. All other types of regions may be drawn only on a scatter plot.

A region can be copied to another graph in the same file or other open files. Regions may also be copied from one instance of the IDEAS application to another.

When you draw a region on a histogram or scatter plot, you create a population of objects defined by the region that may be viewed in the Image Gallery or on other graphs.

To change the attributes of a region or delete a region and the populations dependent on that region see Using the Region Manager .

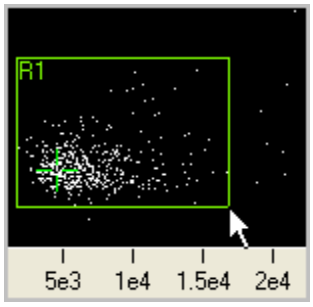
To draw a region on a Scatter Plot

On the Analysis Area toolbar, click either the:

- Rectangle Region, or
- Oval Region, or
- Polygon Region button on the Analysis Area toolbar.

1 The Rectangle and Oval tools work by clicking on the graph at the point where you would like to start the region, and drag to the region endpoint.

The region grows as you drag.



2 Click again to complete the region.

If you are drawing a region on a histogram or scatter plot, the **Create a Region** window appears.

3 Name the region.

4 Click the colored box to select an alternate color.

5 Select **Use for statistics only** if you do not want to create a population from this region.

6 Click **OK**.

The region appears on the graph with the name and color that you selected.

Polygon Tool Option

1 The Polygon tool works by clicking the scatter plot at the point where you would like to start the polygon.

2 Click once for each vertex of the polygon.

3 Double-click to complete the drawing of the region.

A window appears that allows you to name the population created by the polygon region and to assign the region's display properties.

4 Click **OK**.

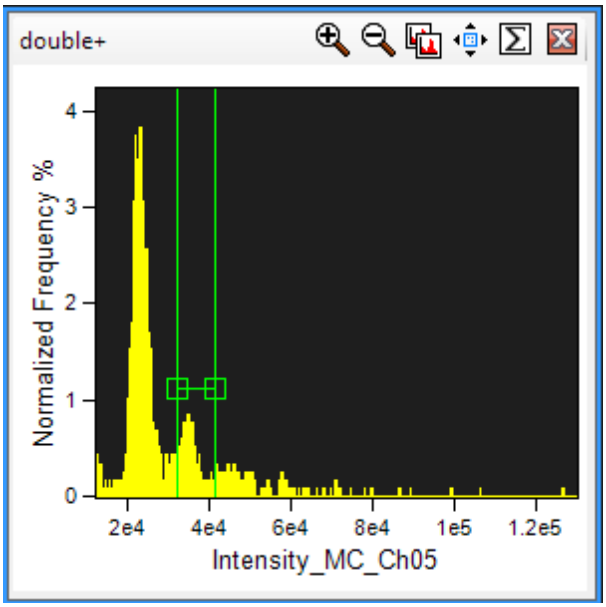
The region appears on the graph with the name and color that you selected.

Tip: Before you click **OK**, you can click **Cancel** or you can click the Pointer button on the Analysis Area toolbar if you decide not to create the region.

To Draw a region on a Histogram

1 On the Analysis Area toolbar, click the Line Region tool.

2 Drag the line across the histogram.



To move or resize a region on a graph

1 Click the Move/Resize Region toolbar button on the graph panel toolbar.

2 Click the region that you would like to move or resize.

When the region is selected, squares that can be moved appear at the vertices and the label.

3 The first time that you drag the region, the entire region and label move.

4 Dragging a specific vertex or label moves only that vertex or label.

5 To finish moving or resizing the regions on the graph, click the Move/Resize Region toolbar button again to deactivate the tool.

The populations and statistics are updated, and the Move/Resize Region toolbar button is deactivated.

Note: The recalculation of statistics and populations may take a moment if the data file is large or if many populations are dependent on the regions that are being moved or resized.

To zoom in on the scale of a graph

1 Click the Scaling toolbar button on the graph panel toolbar.

2 Click and drag to define a rectangular region for rescaling.

The Zoom Out Scaling toolbar button appears in the graph panel toolbar, next to the Scaling toolbar button.

3 Click the Zoom Out Scaling toolbar button to automatically scale the graph.

The Zoom Out Scaling toolbar button is removed from the graph panel toolbar.

To resize a graph

- Select the graph(s) to be resized and then click the sizing button tool small, medium or large.
- A graph may be resized by dragging the right, bottom or lower,right corner.

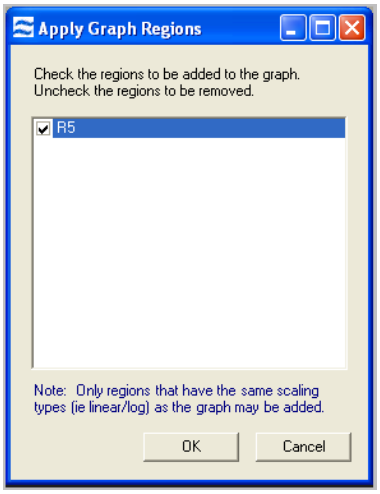
  Tip: Select multiple graphs to make them all the same size.

To copy and paste a region to another graph

1 Right-click anywhere on a graph, and click **Copy Region to Clipboard** on the graph context menu that appears.
  The **Copy a Region to the Clipboard** window appears.
2 Click the region to copy in the list, and click **OK**.
3 Right-click on the graph where you want to paste the region, and click **Paste Region from Clipboard** on the graph context menu that appears.
4 If the region already exists (in other words, you are copying it within the same instance of the application), the **Create a Region** window appears.
5 Rename the region and set the display properties for the resulting new population, and click **OK**.
  Note: When you copy a region, the scale is copied and is no longer associated with the feature from which it was originally drawn. Therefore, the region might not fit on the new graph.

To Apply or Remove a region on a graph

1 Right-click anywhere on the graph, and click **Apply/Remove Region** on the graph context menu that appears. The **Apply Graph Regions** window appears.


Apply Graph Regions
Check the regions to be added to the graph.
Uncheck the regions to be removed.
R5
Note: Only regions that have the same scaling types (ie linear/log) as the graph may be added.
OK
Cancel

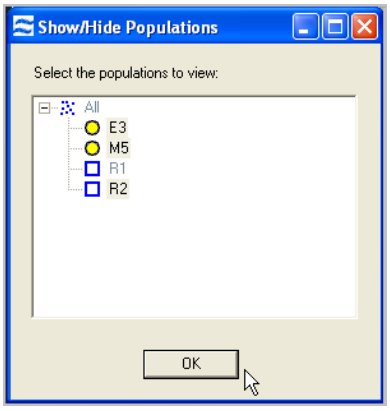
2 Select the regions that you want to appear on the graph.
3 Clear the regions that you want to remove from the graph.
4 Click **OK**.

To show or hide a population on a scatter plot

1 Click **Show/Hide Populations** on the graph context menu.
  The **Show/Hide Populations** window appears.

2 Select the populations that you want to appear on the graph.

3 Clear the populations that you want to remove from the graph.



4 Click **OK**.

Tip: On a scatter plot, you may show or hide any population on the graph–regardless of the features on the axes. Each scatter plot has an original, or base, population. When you show a population on a scatter plot, only those objects that are also in the base population will be shown. To aid in the identification of the populations shown, change the characteristics of the population(s) in the population manager.

Analyzing Individual Images

To analyze an image in more detail, place the image in the Analysis Area to view pixel positions and intensities as well as generate statistics for an area of the image. You can also show the Measurement tool for the image.

Image panels, which are shown in the following figure, each contain a toolbar in the upper-right corner and a context menu that appears when you right-click an image. An image in the Analysis Area is three times the size of an image in the Image Gallery.

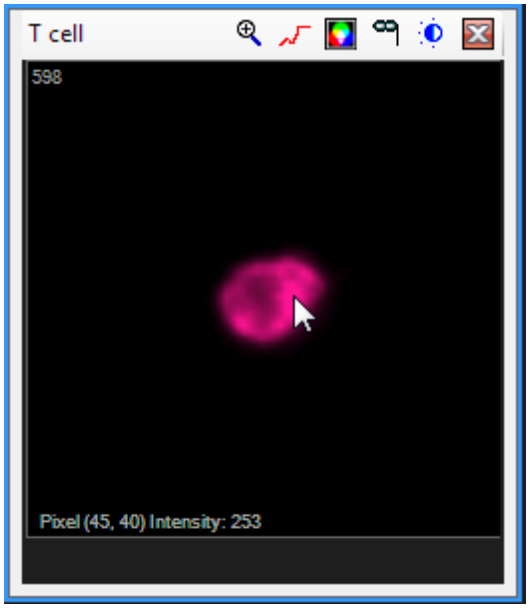
To add an image panel to the Analysis Area

- Right-click an image in the Image Gallery or Analysis Area, and click **Add Image to Analysis Area**on the context menu that appears.

  The image panel appears in the Analysis Area.

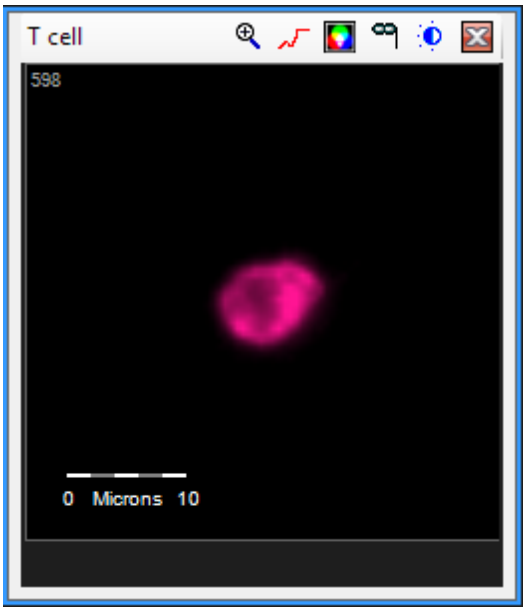
To view the individual pixel intensities of a single channel image

- Move the mouse pointer across the image.
  The pixel positions and intensities appear under the image. (The pixel (0, 0) is positioned at the upper left of the image.)



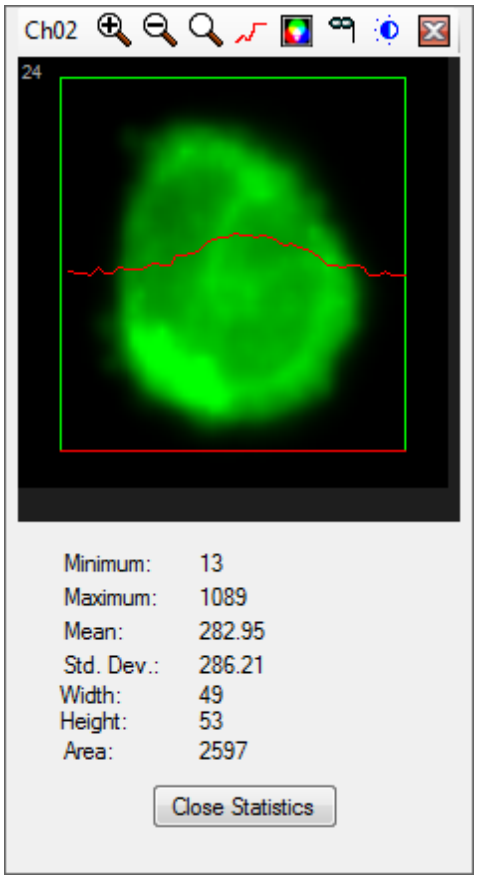
To display the Measurement tool in an image panel

- Right-click the image panel, and click **Show Measurement Tool** on the context menu that appears.
  The 10-micron bar appears.



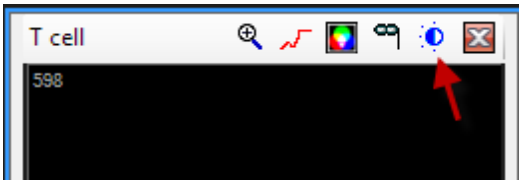
To examine a line profile or the statistics for an area of an image

- While holding the Ctrl key, click and drag to create a boxed area on the image. The **Image Statistics** are shown in the image panel. The statistics are calculated for the area that is defined by the box. The line profile (the wavy line in the image panel) represents the pixel intensity at each position along the red line of the box.



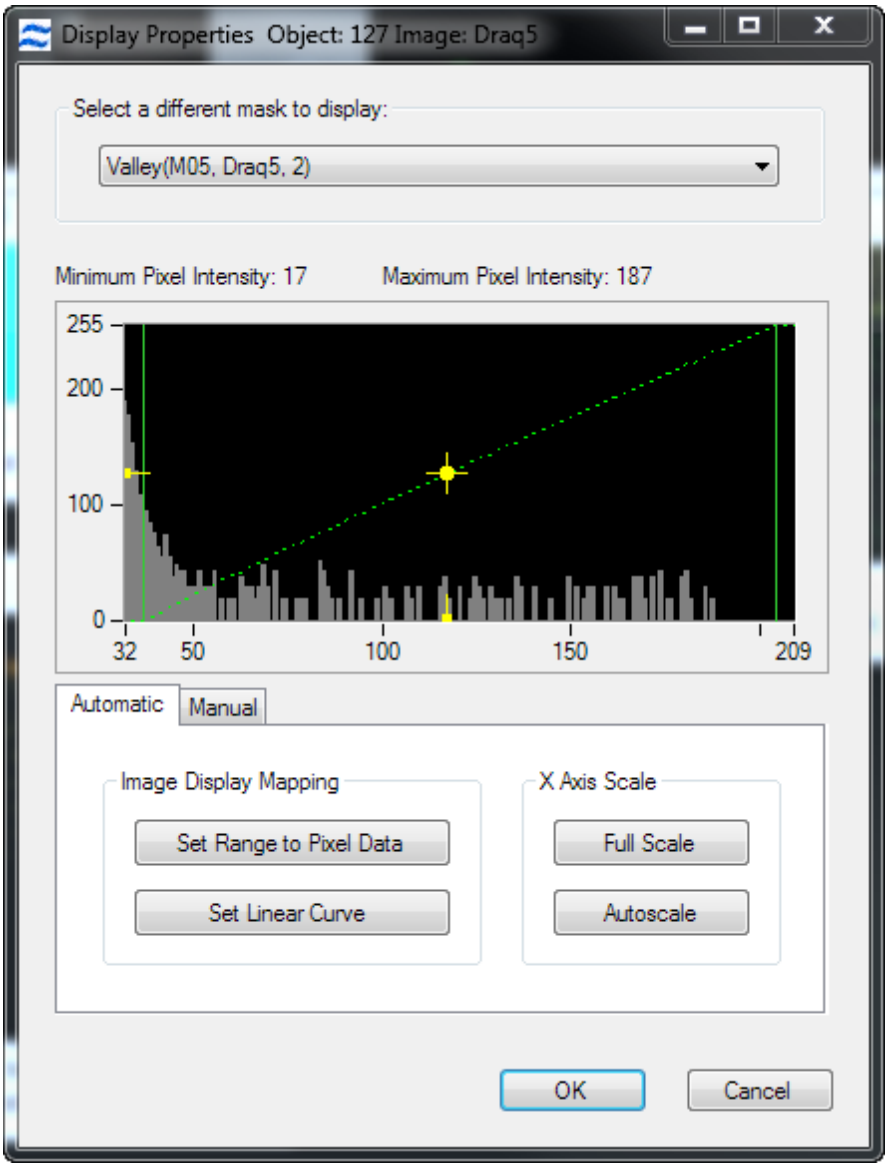
To change the display properties of an image

1 Click the Channel Display Properties button on the image panel toolbar.

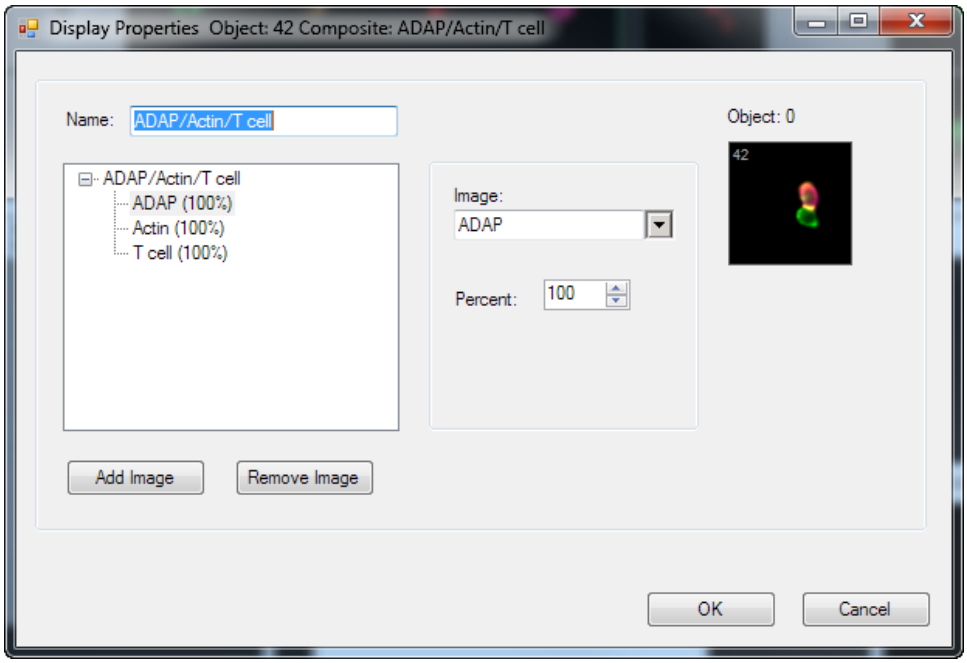


The **Display Properties** window appears.

- For single channel image, you can change the displayed mask and adjust the display intensity mapping. For more information, see Setting the Image Gallery Properties.



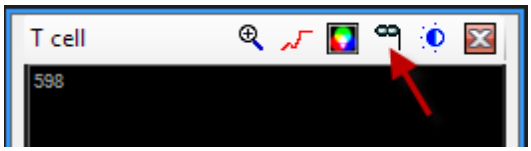
- For a composite image, you can change the images in the composite and adjust the percent contribution of each image, see Setting the Image Gallery Properties.



2 Click **OK**.

To show or hide the mask for a single channel image

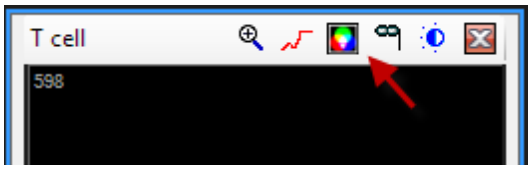
- Click the Mask button on the image panel toolbar, or right-click the image and then click **Show/Hide Mask** on the image context menu.



The mask appears as a transparent cyan overlay on the image.

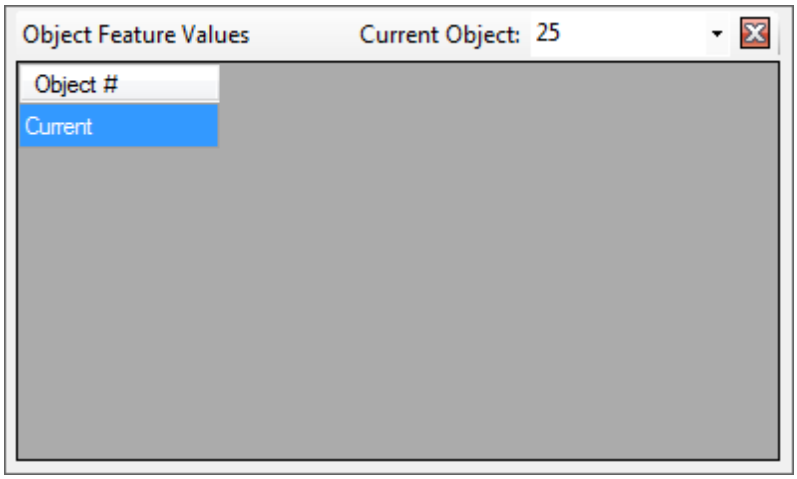
To turn the color on or off

- Click the Color button on the image panel toolbar, or right-click the image and then click **Color Off** or **Color On**.



Viewing the Object Feature Values

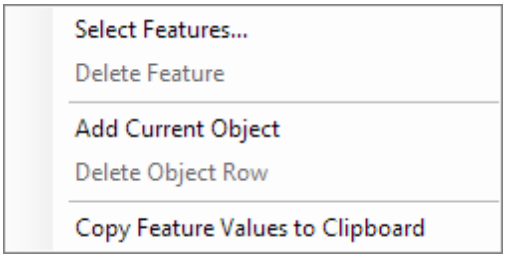
The **Object Feature Values** table, which is shown in the following figure, displays a selected set of feature values for selected objects. For each feature, the name, value, and description are shown.



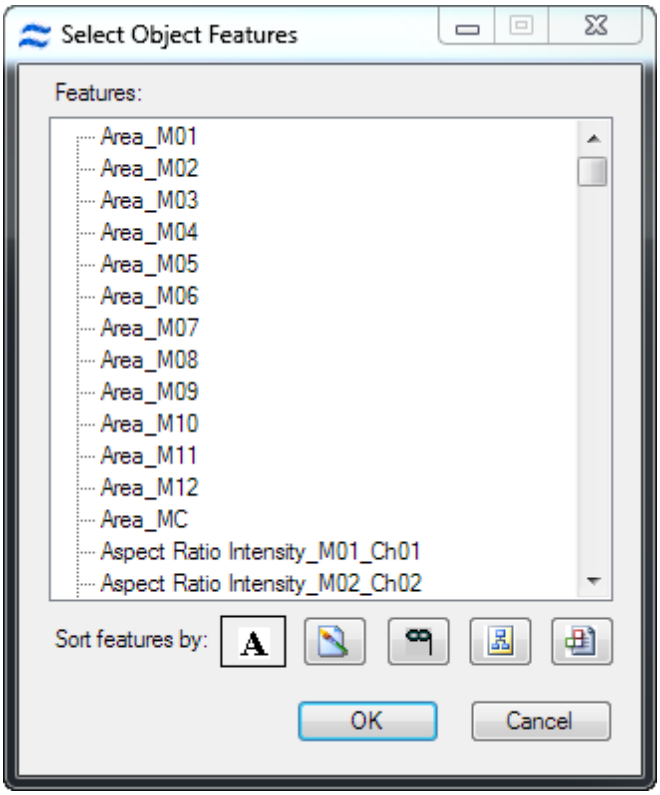
To view and customize the features shown in the Object Data table

1 Click the **Object Feature Values** tool.

2 Right-click anywhere in the table area to open the menu.



3 Choose **Select Features**.
The **Select Object Features** window appears.



4 Select the features to view. Multiple features may be chosen by holding down the Ctrl key.

5 Click **OK**.

The features appear in the Object Data table.

6 To add selected objects to the table right-click and choose **Add Current Object**.

To export or copy feature values

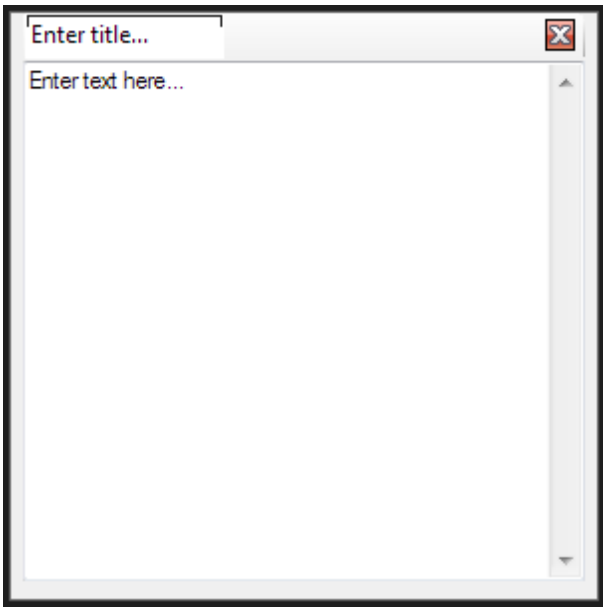
- Right-click in the table and choose **Copy feature values to clipboard**.

Adding Text to the Analysis Area

To add text to the Analysis Area

1 Click the Text button on the Analysis Area toolbar.

A text panel is added to the analysis area.



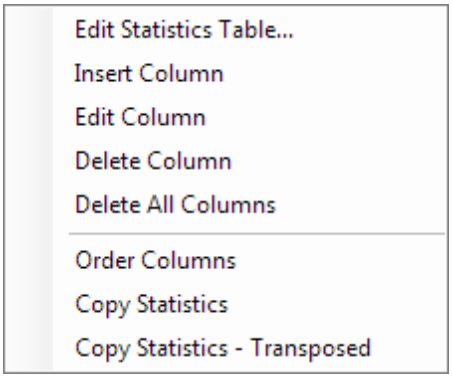
2 Type a title and text.

Population Statistics

The **Population Statistics** table displays selected statistics for chosen populations. The statistics that are supported are the Count, Percent Total, Percent Gated, Percent, Concentration (count/sample volume), Mean, Median, Standard Deviation, MAD (Median Average Deviation) RD -Mean, RD -Median, CV, Minimum, Maximum, Geometric Mean, Mode, variance, and NaN (not a number).

To view and customize the population statistics

1 Click the **Populations Statistics** tool.
2 Click the + next to the population to expand the list of populations.
3 Columns can be moved by click-dragging.
4 Right-click in the grey area in the table or on a column heading and the menu opens.



5 **Edit Statistic Table** opens a Statistics Properties window to enable changes to multiple column statistics.
6 To add a single statistic column select **Insert Column**.

7 Select **Edit Column** to make a change.

8 To delete a single column right-click on that column and select **Delete Column**.

9 Select **Delete All Columns** to clear all statistics.

10 **Order Columns** places the columns in default order.

11 **Copy Statistics** copies the selected rows of the table in a text format that can be pasted into other programs such as Excel.

12 **Copy Statistics - Transposed** copies the selected rows of the table and transposes the data so that when pasted into other programs such as Excel the rows become columns.

Using the Feature Manager

This section describes how to create and delete features and to create multiple features by selecting categories. The only new feature options for FlowSight basic files without QI are combined features. The following subsections cover this information:

Viewing feature definitions

Creating New Features with the Feature Manager

Ranking features by discriminating power

Overview of the Feature Manager

The IDEAS application defines a set of base features that you can use to create features for each object. To do so, you use the object's mask and/or its channel images. After a feature has been created and its value calculated for all cells, you can plot the feature values or view them as statistics for any population. For descriptions of all the base features, see Understanding the IDEAS® Features and Masks.

When the IDEAS application opens a .cif or .rif file, the application calculates the values of features as defined by the selected template. You can refine your template so that it includes only those features of interest for your experiment.

You use the Feature Manager to examine existing features and to define new ones. To gain access to the Feature Manager, select **Analysis** > **Features** or select it from one of the context menus that are available in the histogram and scatter plot panels with a right-click. While the Feature Manager is open, all calculations for creating graphs and statistics are disabled. However, you may view images and change the population and channel views. When you close the Feature Manager, any changes to feature names, definitions, and values are reflected in any currently displayed graphs and statistics. The values of newly created features are also calculated at this time.

You can create single features and combined features. You create a single feature by selecting a base feature, such as Area or Intensity, along with a mask and/or an image. This option is not available for basic FlowSight files without the Quantitative Imaging (QI) upgrade.You can create a combined feature by defining a mathematical expression that includes one or more single features that exist in the feature list. FlowSight files without the QI upgrade can utilize the combined feature option.

Some features, such as Area, depend on the boundary of a cell. These features require you to select a mask that defines the portion of the image to use for the calculation. Other features, such as Max Pixel, depend on pixel intensity measurements and require you to select an image. Other features require you to select a mask and one or more images.

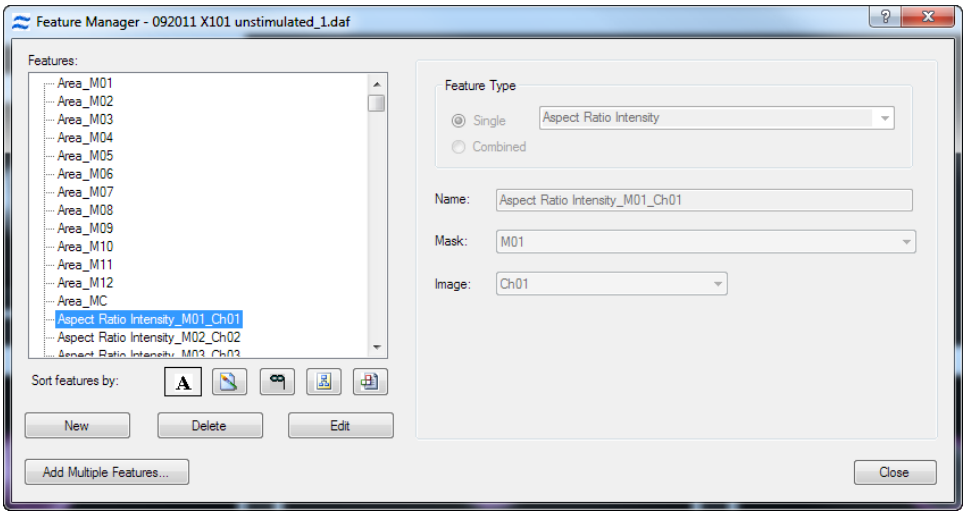
You can add and remove features from the feature list. The feature definitions are stored in templates, so the definitions are available when you analyze multiple data

files. The default template used for ImageStream data or QI FlowSight data includes most of the base features for each channel image and channel mask that the feature list contains. Certain features, such as Similarity and Spot, require extensive calculations so the default template does not include them. The reason is to save time when you load files. However, you can add these features to the feature list.

Viewing feature definitions

To view existing features

1 Click **Analysis** > **Features** or select Features from a graph panel context menu. The **Feature Manager** window appears.



2 Choose an icon to sort the features:

Table 1: Sorting Features

Feature Icon	Definition
	Sorts features alphabetically.
	Sorts features based on the images used.
	Sorts features based on the masks used.
	Sorts features by category, such as size, location, shape, texture, signal strength, and system.
	Sorts by base features, such as area, aspect ratio, intensity, and object number.

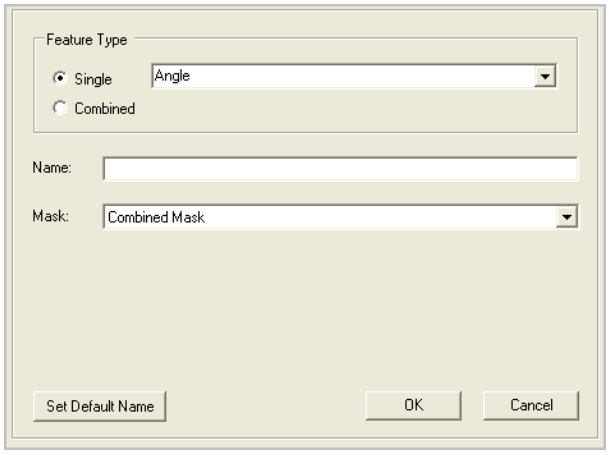
3 Click a feature in the **Features** list to view its definition in the right side of the window.

Creating New Features with the Feature Manager

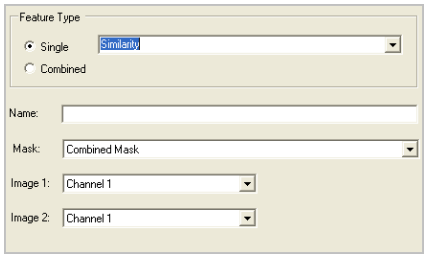
To create a new single feature

A single feature uses the definitions of a base feature along with a mask and/or an image.

1 Click **New** in the Feature Manager.
The right-hand area of the Feature Manager is enabled.



2 Select **Single** as the **Feature Type**.
The **Mask** and **Image** lists become visible depending on the single feature selected.

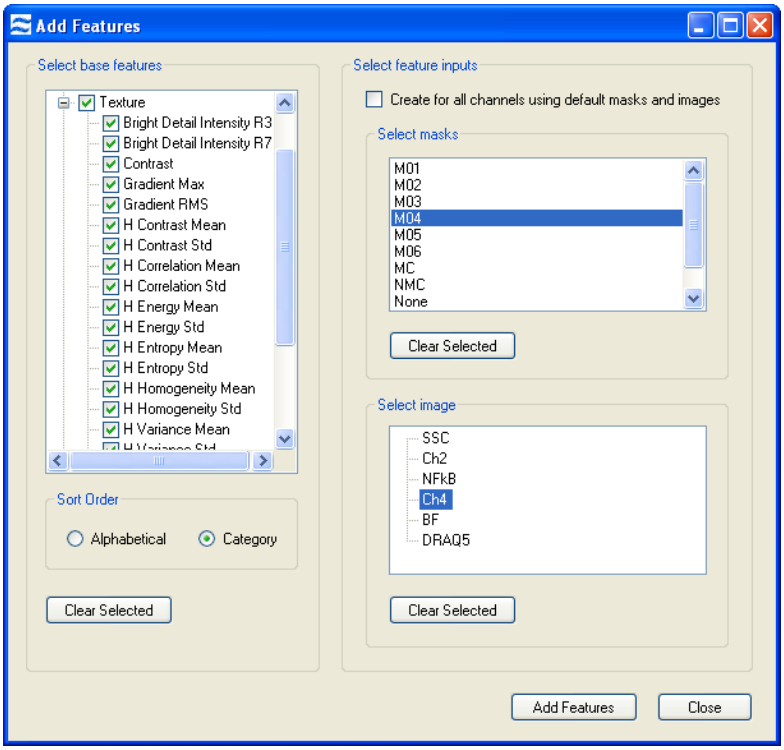


3 Select the mask and/or image that you want.
4 Enter a unique feature name or click **Set Default Name**.
The default name is the name of the base feature followed by the name of the mask and name(s) of the image(s).
5 Click **OK** to add the new feature.
It appears in the **Features** list on the left side of the Feature Manager.
6 Click **Close**.
Note: When you close the Feature Manager, the IDEAS application calculates values for the new features. These calculations may take several minutes, depending on the number and complexity of the new features and the size of the image file.

To create multiple features

1 Click **Add Multiple Features** in the Feature Manager.
2 Sort the feature list alphabetically or categorically.
3 Select multiple base features and masks

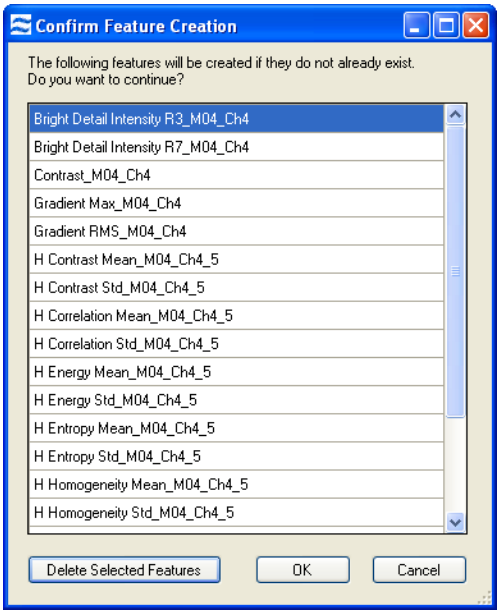
4 Select one image or check the box to create for all channels using default masks and images.



5 Any list can be cleared by clicking the Clear Selected button.

6 When finished click **Add Features** to add the new features to the list.

7 Confirm the features in the next window.



8 Delete any features you do not want to calculate.

9 Click OK when finished. The new features are added to the list in the feature manager.

10 Close the Add Features window.

11 Close the Feature Manager. The new features are calculated when the feature manager closes.

To create a new combined feature

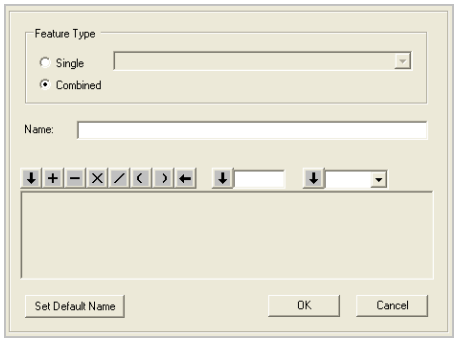
A combined feature uses one or more single features created by a mathematical expression.

1 Click **New** in the Feature Manager.

The right hand area of the Feature Manager is enabled.

2 Select **Combined** as the **Feature Type**:

The editing interface appears.



3 Enter the feature name in the **Name** box or use **Set Default Name** after you have created your expression. The default name is the name of the definition created.

4 Use the toolbar to build a definition (mathematical expression) of features and operators:

Table 1: Combined Feature Tasks and Toolbar

Task	Toolbar
Add a feature to the definition.	Double-click the feature in the **Features** list. Or, single click the feature in the **Features** list and select click the leftmost down-arrow button on the toolbar.
Add an operator or a parenthesis to the definition.	Click the corresponding button on the toolbar.
Add a number to the definition.	Enter the number in the box and then click the corresponding down-arrow button. 3.14 If the area is greyed-out, an operator must be selected first.
Add a function to the definition.	Select the function in the list and then click the corresponding down-arrow button. ABS COS SIN SQR SQRT The available functions are ABS (absolute), COS (cosine), SIN (sine), SQR (square), and

Task	Toolbar
	SQRT (square root). If the area is greyed-out, an operator must be selected first.
Remove an item from the end of the definition.	Click the left-arrow button on the toolbar.

5 Click **OK**.

6 Click **Close**.

Note: When you close the Feature Manager, the IDEAS application calculates values for the new features. These calculations may take several minutes, depending on the number and complexity of the new features and the size of the image file.

To delete a feature

1 Select one or more features in the **Features** list by clicking them. To select more than one feature, use the Ctrl key.

2 Click **Delete**.

A warning message will confirm or cancel deletion.

Note: Deleting a feature also deletes any populations that are dependent on that feature. Your feature list may become large and unwieldy. You can narrow down the list without deletions by sorting the list. See Using the Feature Manager for more information.

Ranking features by discriminating power

With the IDEAS application you are able to create an unlimited set of features by using the Mask manager to define location and the Feature manager to choose a mathematical expression that uses the image pixel data and/or the mask. This can make it difficult to choose a feature that provides good statistical separation of populations of cells that have different appearances from each other. The following procedure describes the manual process to find features that separate two populations of cells from each other with minimal knowledge of the feature set. A general description of the steps is followed by a specific example.

Note: IDEAS 6.0 includes the 'Feature Finder' wizard which replaces this manual process. This process may still be useful for making features that are not included in the feature finder template. See theFeature Finder Wizard for more details.

General:

1 Set image display and draw preliminary regions to include cells of interest (i.e. single, focused, positive cells).

2 Visually inspect overall quality of images and experiment to determine whether to proceed or redo the experiment.

3 Create two tagged ‘truth’ populations of cells that represent the phenotypes you wish to discriminate. Perform the discrimination on one characteristic difference at a time.

4 Create any additional masks and features you think may help differentiate the truth populations.

5 Calculate the statistical discrimination (RD) between the two populations afforded by features in 1 category at a time. Pick the top feature for each category.
6 Plot the features with the highest RD for the truth populations for each category
7 Validate by applying the feature to the base population, independent controls if available and on multiple files and experiments.

Example:

Treatment-induced actin polarization. The data file is available for practice. Log in to your account on the Amnis website and look in the folder 'Training data files'.

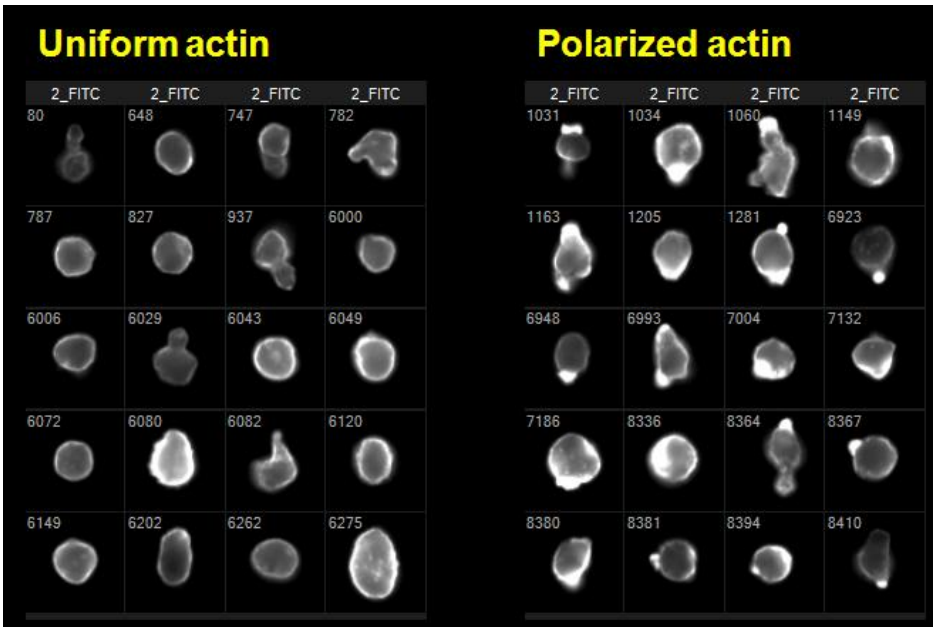
- Cells were incubated with inducing compound for 1 hour.
- The nucleus was probed with DAPI and actin stained with FITC.
- Large event image files were collected on the ImageStream.
- Compensation and analysis was done in IDEAS.

The following steps find the best features that distinguish changes in actin distribution.

1 Gate single, focused, actin-positive cells. View cells of interest.
2 Create the truth populations from within the cells of interest using the tagging tool.

   Note: If truth populations are in different files, merge them together before beginning. When selecting truth populations, choose images that represent the full phenotypic range of each truth. In this example case, note that the 'uniform actin' truth population contains cells of varying shape and intensity that all have uniform actin distribution. Bias introduced during the selection of truth populations will likely also bias the outcome during statistical ranking.

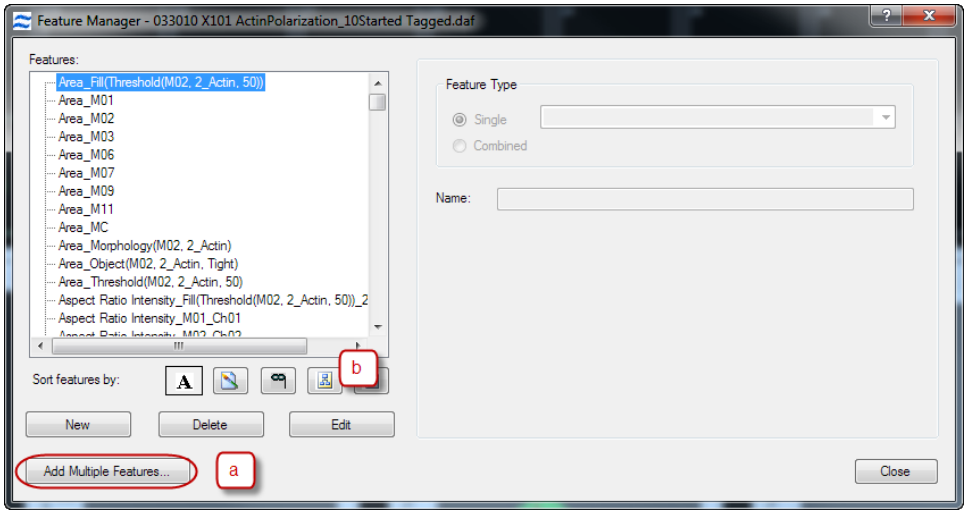
The following figure shows the truth populations chosen to find a feature to discriminate uniform versus polarized actin:



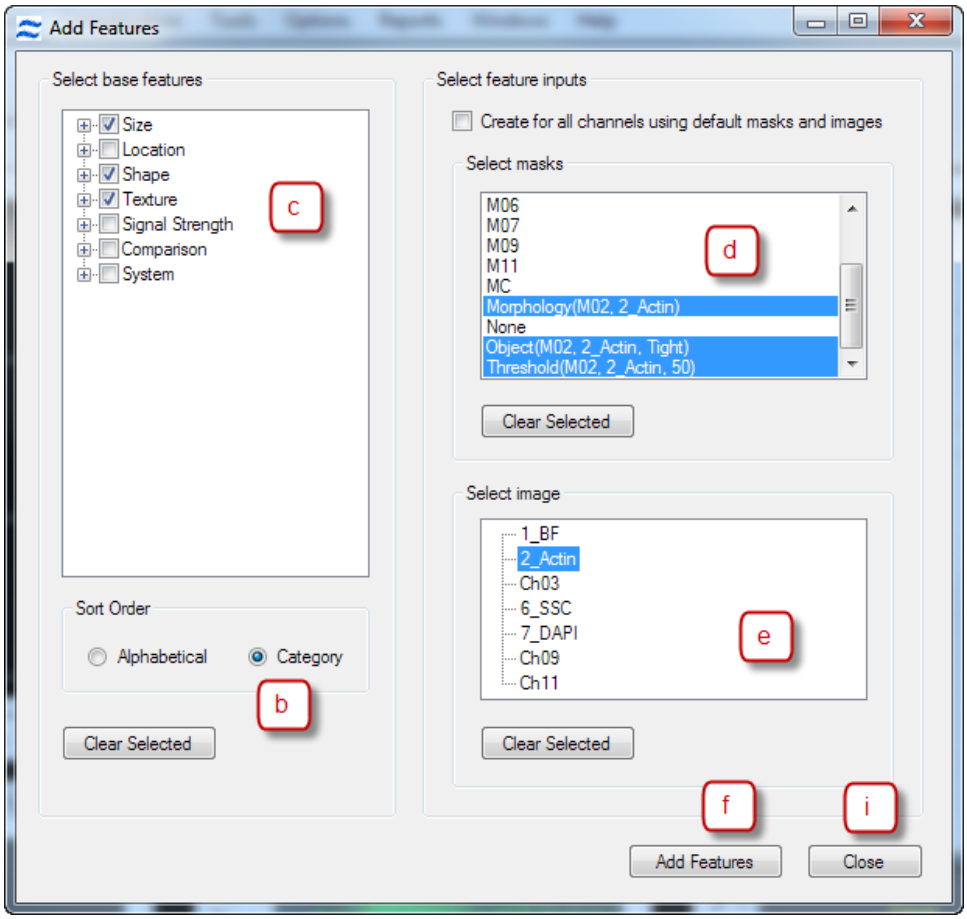
3 Create the Morphology and one or moreThreshold masks for the actin image.

4 Create features from the Size, Shape, and Texture categories using the Morphology, Threshold and Default actin channel masks.

a. Choose Features from the Analysis menu and click Add Multiple Features.



b. In the Add Features window select Category as the 'Sort Order'.



c. Check Size, Shape and Texture base feature boxes.
d. Select the actin masks (Morphology, Object, Threshold, M02)
e. Select the actin image (FITC).
f. Click Add Features to display the list of features to add.
g.In the next window Click OK to add the features.Features that already exist will not be recalculated.
h. Click OK and Click Close.
i. Close the Feature Manager by clicking Close and the features will be calculated.

5 Add the feature statistics to the population statistics table. Do this one category at a time. Multiple statistics tables can be added to the analysis area, one for each category of features.

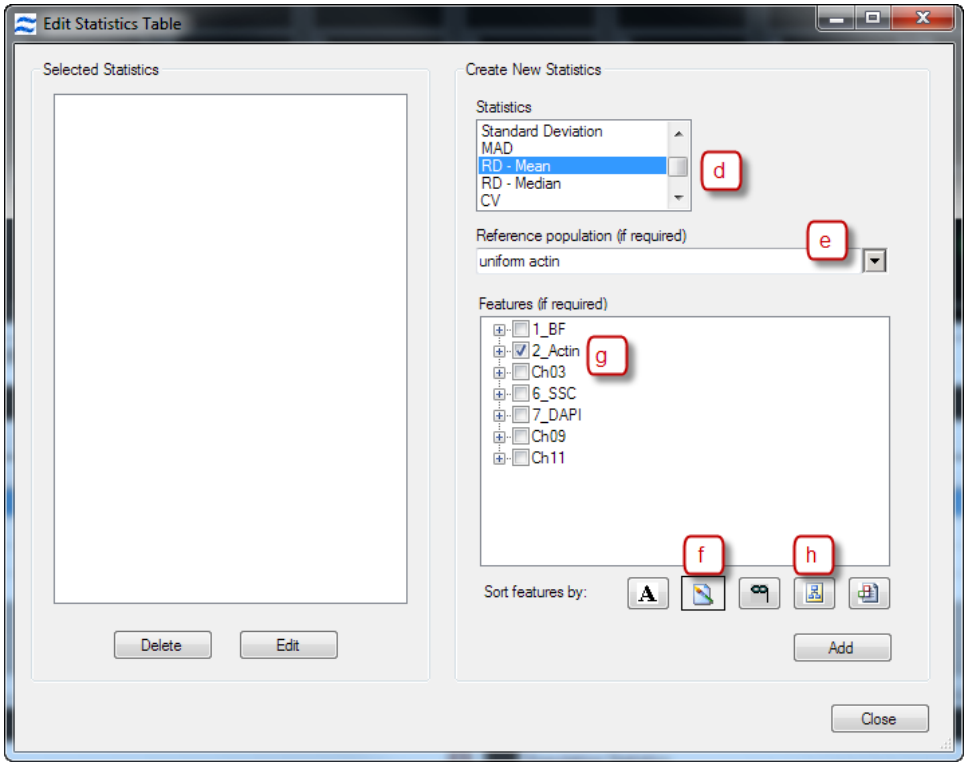
Once the features are calculated you can use the RD,(Fischer's Discriminant Ratio) to a statistics table. The RD measures the separation between 2 populations. In this case, the 2 truth populations picked in step 2. In

order to get the statistic for 1 category at a time, select all of the features for the image and then deselect categories, to leave 1 category for the channel selected.

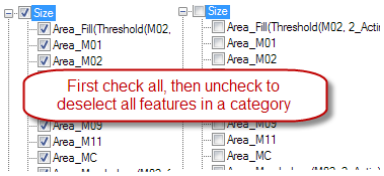
a. Click on Σ to add a statistics table to the analysis area.
b. Right-click in the table and choose Edit Statistics Table.
c. Delete any statistics from the list.
d. Select the statistic 'RD -Mean'.
e. Select one of the truth populations in the Reference population box.

f. Sort by Images Used by clicking on the icon .
g. Check the box for the Ch02/Actin image.

h. Sort the features by Category .



i. De-select all but 1 category by checking and unchecking the box for the categories you want to de-select. Note that the box next to the category will be checked only if all of the features (all channels) in the category are selected.



j. Click 'Add Statistics'.
k. Click Close.
l. Repeat until each statistics table contains 1 category of features for Ch02(Actin).

6 Launch Excel and then Copy and Paste the statistics into the excel spreadsheet.

a. Select the row of statistics for the 2nd truth population (the one not chosen above).
b. Right-click in the statistics table and choose Copy Statistics - Transposed.
c. Paste into an Excel spreadsheet.
d. Keep all of the features and values selected and sort the data set on the values column (heading may be the population name) largest to smallest. The feature with the largest RD will be at the top.
Note: you may have NaN values for some of the features. This means Not a Number and occurs in some cases when there is a division by 0. These can be ignored.

7 Validate the features in IDEAS. Plot the features with the highest RD for the truth populations and draw regions to discriminate.

8 Apply regions to the base population, independent controls if available and on multiple files and experiments.

9 Look for false negative and positive cells.

10 Repeat process if necessary by refining/creating new truth populations.

NOTES ON EVALUATING THE FEATURES:

Consider the features that produce the highest Rd. If there are any intensity based features make sure that the staining was not uneven due to technical issues. If it is a size feature, does it make sense with what you know about the cells and biology of your experimental system? Since the feature value ranges vary between features this is an approximate comparison and the result should be validated by viewing images across the feature range from the whole population.

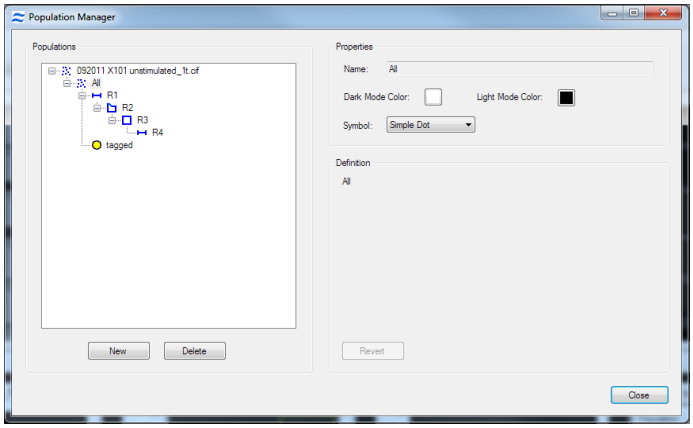
Using the Population Manager

A population is a group of objects. You create populations by drawing regions on graphs, by hand-selecting (tagging) objects in the Image Gallery or on plots, or by combining existing populations. After a population has been defined, you can view it in the Image Gallery or on a plot and you can use it to calculate statistics.

The Population Manager provides a central place for maintaining the display properties of existing populations and for creating new combined populations.

To open the Population Manager and view the population definitions

1 Select **Analysis** > **Populations** or right click a graph and select **Populations**. The Population Manager window appears.



Note: The list of populations is presented as a hierarchy that shows the dependencies of the populations on each other. The icon associated with a population indicates how the population is defined.

icon	Defined by
	Tagging
	Region
	Combined

The definition of a selected population is shown in the Definition area.

To edit the display properties of a population

1 Within the **Population Manager**, click a population in the **Populations** list.
2 Change the name in the **Name** box.
3 Click a **Color** square to select a new color on the color palette and click **OK**.
4 Click a display symbol in the **Symbol** drop down menu.
5 Click **Close** to save the population changes.
6 Click **Revert** to reject the changes.

To delete a population

1 Within the **Population Manager**, click a population in the **Populations** list.

2 Click **Delete**.

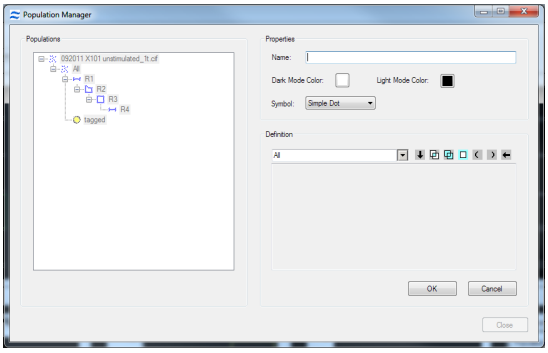
A confirmation warning message appears indicating all the dependent populations that will also be deleted.

3 Click **Yes** to confirm.

To create a new combined population

1 Within the **Population Manager** (**Analysis** > **Populations**), click **New**.

The right side of the Population Manager window changes to allow you to define a new population.



2 Enter a unique population name in the **Name** box.

3 Click a **Color** square to select a new color on the color palette and click **OK**.

4 Click a display symbol in the **Symbol** drop down menu.

5 Use the toolbar to build the population definition as described in the table and click OK when done:

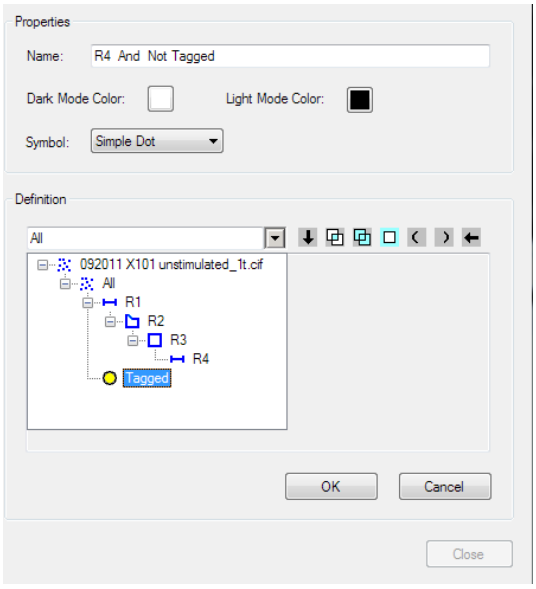


Table 1: Population Tasks and Toolbar

Task	Toolbar
Add a population to the definition.	Select the population from the drop-down menu.

Task	Toolbar
Combine two populations.	Use the Boolean AND or OR operator. Use the AND operator to include only the objects that are in both of the original populations. Use the OR operator to include the objects that are in either one of the original populations.
Select objects that are not in the original population.	Use the Boolean NOT operator. The NOT operator specifies which population will not be used. Note: you must use AND before NOT.
Affect the order of operations.	Use the parentheses toolbar buttons.
Remove an item from the end of the definition.	Click the left-arrow button on the toolbar.

See Creating Tagged Populations for information about tagged populations.

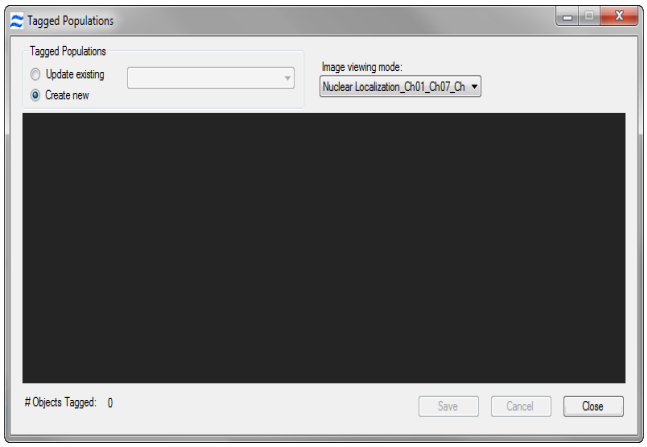
Creating Tagged Populations

You can hand-select objects from either the Image Gallery or a graph and group them into a population.

To create a hand-selected population

1 Click the Tagging Mode toolbar button to begin.

The **Tagged Population** window appears.



2 Select either **Update existing** or **Create New.**

- To **Create New**, double-click images within the Image Gallery and select **Save**. Enter a new population name. Each population is given a new color and the symbol solid diamond for ease of viewing in plots.

3 If you selected **Update existing**, choose a population to update in the drop down menu.

4 In the **Image viewing mode** list, choose the mode that you want from the drop down menu. See Setting the Image Gallery Properties for more information.

5 To add or remove an image from the tagged population, double-click either the image in the Image Gallery or a dot in a bivariate plot.

The selected channel image for each tagged cell is displayed in the viewing area of the Tagged Populations window. In the Image Gallery, a small smiley-face icon appears on the left side of each tagged image. Each tagged object is also displayed as a yellow star in a graph in the Analysis Area. The number of objects in the tagged population is updated in the bottom, left corner.

6 If you are updating an existing population, click the **Update** button in the Tagged Populations window.

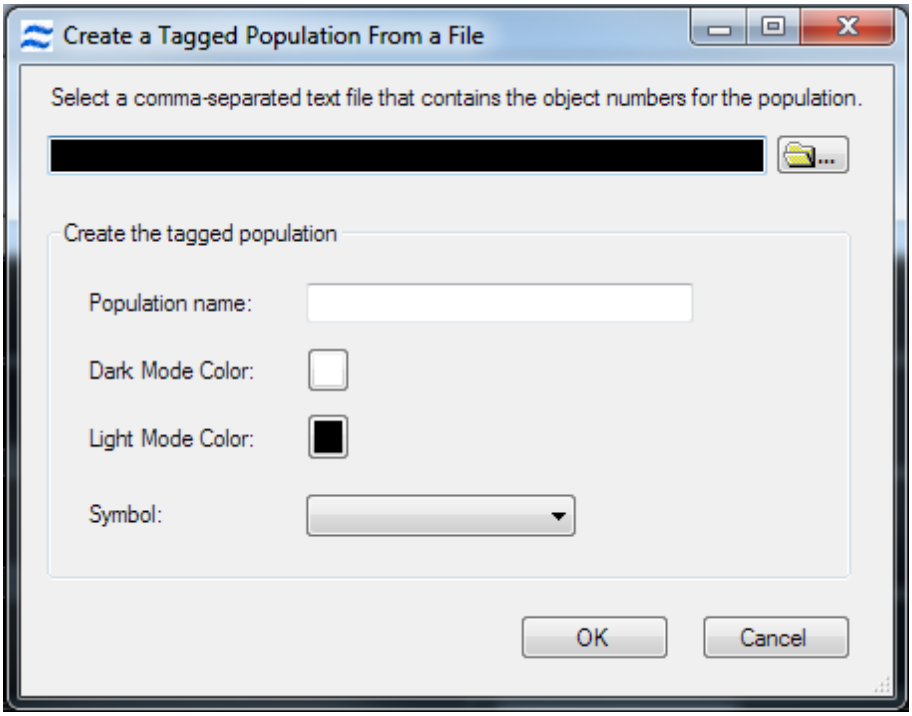
7 When you are finished updating, click **Close** in the Tagged Populations window.

Note: The tagging mode remains open until you click **Close**, and as long as the Image Gallery is in tagging mode, you cannot create, resize, or move any regions on the graphs.

Creating a tagged population from a file of object numbers

You can use a comma-separated text file of object numbers to create a tagged population.

Select Create Tagged Population from File under the Tools menu.



Browse for the file.

Name the population, select the color, symbol and click OK.

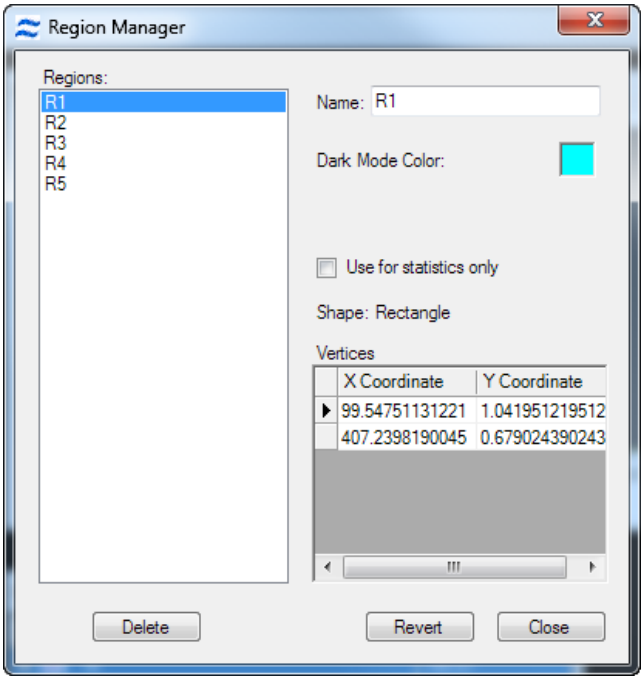
Using the Region Manager

The Region Manager provides a central place for defining the display properties, names, and positions of existing regions. Regions can be deleted in the Region Manager tool.

Regions are drawn on graphs to create new populations, based on the physical location of objects on a graph, and to compute statistics. Tools for drawing regions are found on the Analysis Area toolbar. See Creating Regions on Graphs for more information.

To open the Region Manager and view the region definitions

1 Select **Analysis** > **Regions** or right click a graph and select **Regions**.
The Region Manager window appears. Click on the region in the list you want to view.



To edit a region

1 Within the **Region Manager**, click a region in the **Regions** list.
2 Change the name in the **Name** box.
3 Click a **Color** square to select a new color on the color palette and click **OK**.
4 Change the X or Y position of the vertices in the **Vertices** box.
5 Select or de-select the Use for statistics only box.
6 Click **Delete** to delete a region.
7 Click **Revert** to reject the changes.
8 Click **Close** when finished.

Note: When a region is deleted, all populations that are defined by that region will be deleted. A warning dialog box appears listing the populations that will be deleted.

Creating Reports and Exporting Data

The following six-page sample report was created by copying data from IDEAS, the ImageStream Data Acquistion forms and excel into a MS word document. This template can be found in the customer documents in your training materials or in the knowledge-base of your account on the Amnis website.

Once you have finished analysing an experiment you will want to report the results into third party applications. To streamline the process the first step is to define your application defaults, image gallery settings and statistics report definitions. This section covers the following topics that will help you to create reports.

Viewing and Changing the Application Defaults

Setting the Image Gallery Properties

Prepare the image gallery and analysis area for reporting.

Copy full or partial screens

Copy images to the clipboard or save images to files

Copy graphs to the clipboard or save graphs to a file

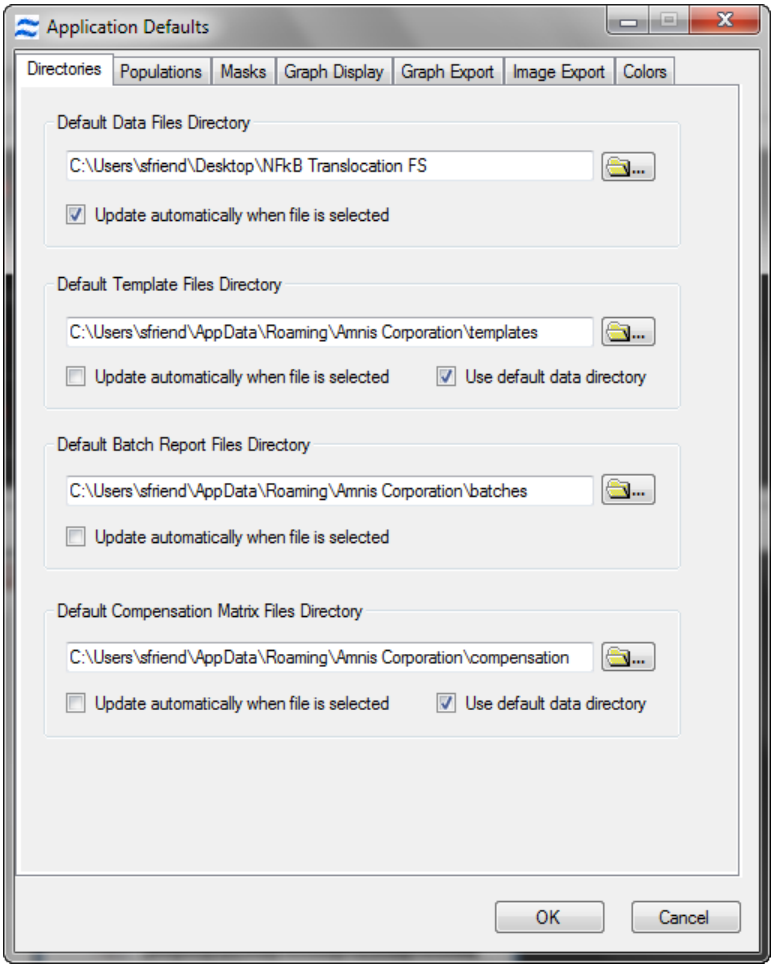
Reporting Statistics

Exporting Data into other analysis applications

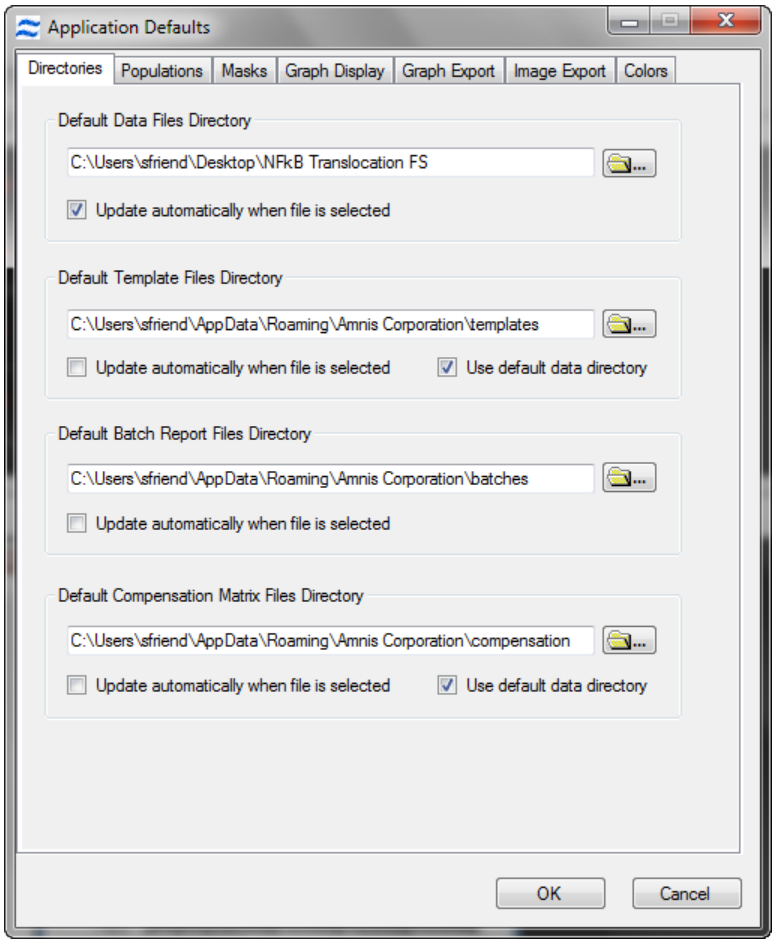
Printing Data

Viewing and Changing the Application Defaults

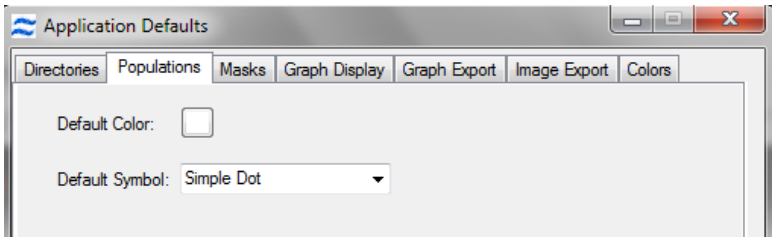
To view or change these defaults, choose**Application Defaults** from the **Options** menu, Each tab allows you to view or change the default settings.



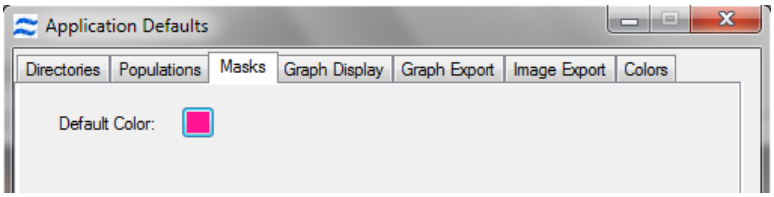
The **Directories** tab contains the default Data, Template, Batch or Compensation Matrix file directories.



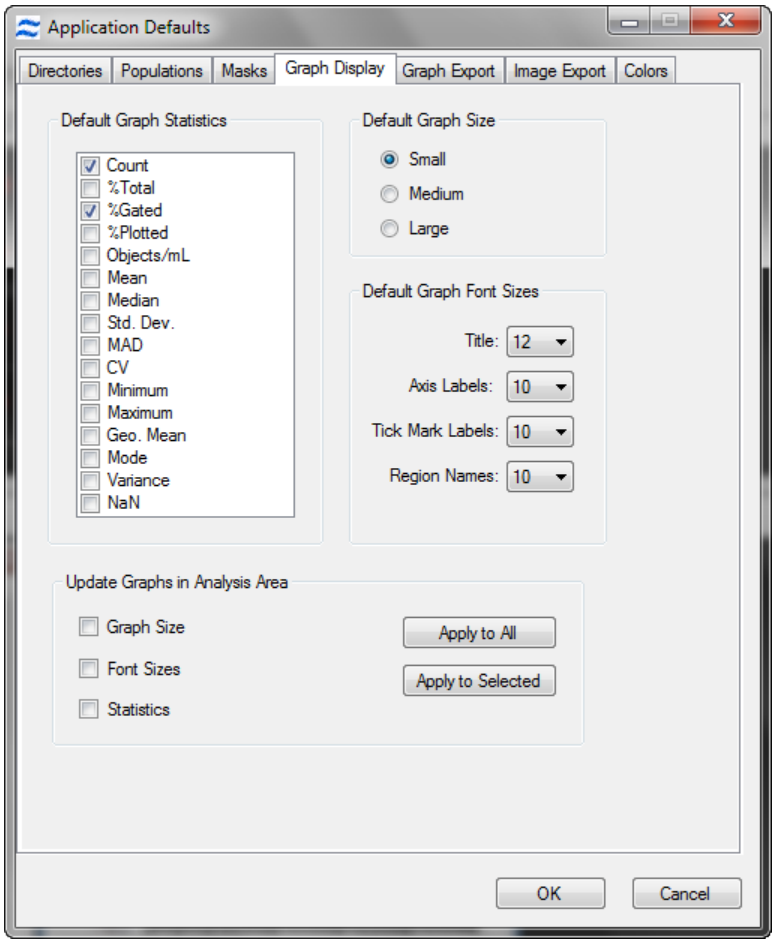
The **Populations** tab contains the default color or symbol for populations. To change the default settings, click on the color to or choose a default symbol from the list.



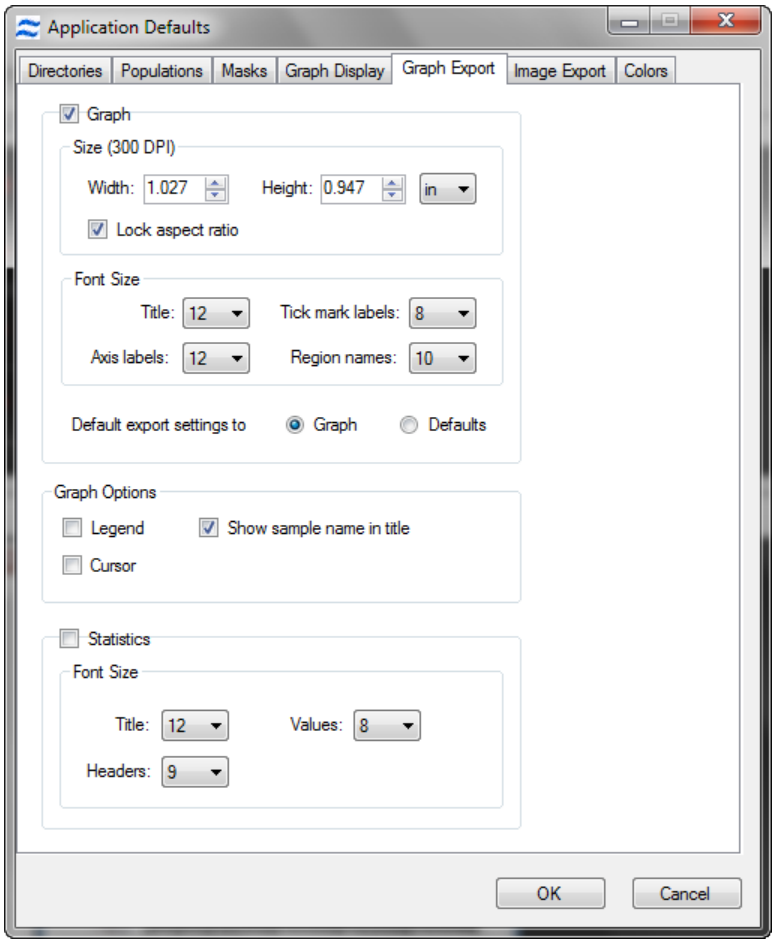
The **Masks** tab contains the default mask color. To change the color of the mask click on the color button.



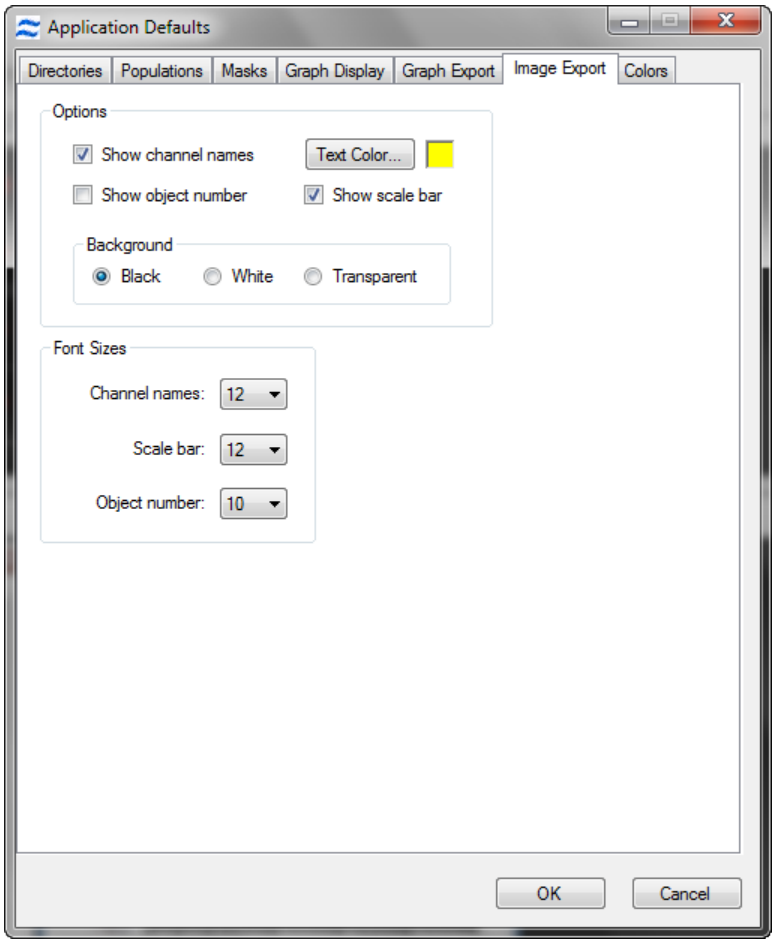
The **Graph Display**tab contains the default list of statistics shown for a graph. Check the box next to the statistic to have it show below the graphs when statistics are shown for a graph. The default graph size and font size for the graphs in the analysis area may be changed in this tab.



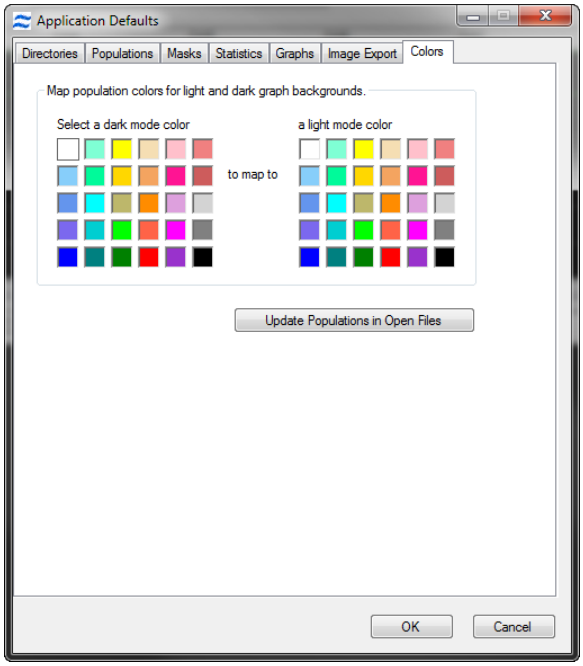
The **Graph Export** tab contains the default settings for printing and exporting graphs when copying and pasting from IDEAS for reporting into other programs.



The **Image Export** tab contains the default settings for image export when copying and pasting from IDEAS for reporting into other programs.



The **Colors** tab contains the mapping of dark and light mode colors.



Setting the Image Gallery Properties

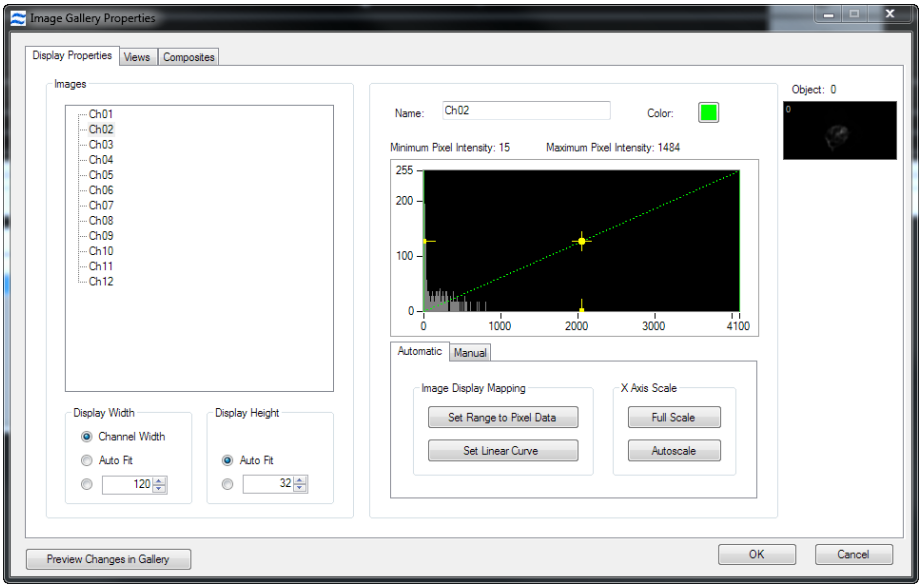
When a new data file opens in the default template, you might find it difficult to clearly see cell morphology because the Image Gallery display properties have not yet been properly adjusted for the data set.
To optimize the display you may use the wizard Display Properties Wizard to set the pixel intensity mapping to the display range. Manual adjustment and other settings are described below.
Clicking the **Image Gallery Properties** toolbar button opens the Image Gallery Properties window, which contains the following tabs:

- **Display Properties**–Allows you to define the name, color, and display intensity mapping for each image. Allows adjustment of the image size for the image gallery.
- **Views**–Allows you to customize the views for the Image Gallery.
- **Composites**–Allows you to create composites and adjust the amount of color from a channel that is included in a composite image.

To customize the Image Gallery display properties

1 Click the **Image Gallery Properties** toolbar button to begin.

The **Image Gallery Properties** window appears with the **Display Properties** tab displayed.



To change the size of the panels in the image gallery

1 Display Width and Display Height can be specified or changed to Auto Fit in the lower left section of this window.

To change the name or color for each image

1 Select an image in the list of images on the **Display Properties** tab of the **Image Gallery Properties** window.

2 On the right side of the window you can type a new, unique name for the selected image. Note that each image is provided with a default name and the image names appear near the top of the Image Gallery.

3 Click the colored square for the selected image.

4 Click the color that you want in the color palette.

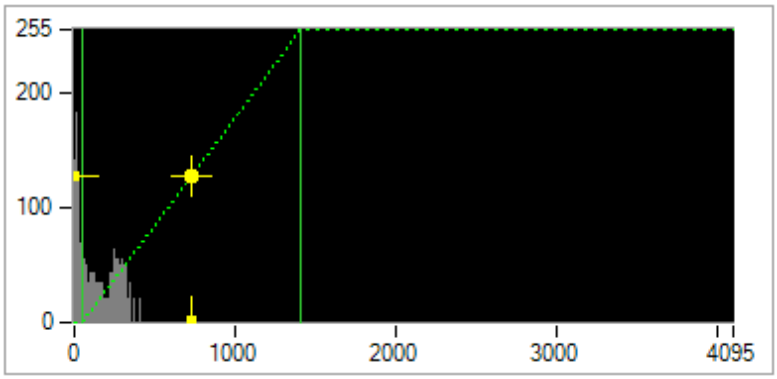
5 Click **OK** to close the palette.

Tip: The grayscale image in each channel is assigned a default color for image display in the gallery. Setting the color to white is equivalent to using the original grayscale image. The colors are also used to build composite images.

To fine-tune the image display intensity for an image

1 On the **Display Properties** tab of the **Image Gallery Properties** window, select an image by clicking the image name in the list. The graph for the currently selected image is shown in the window and updates as the changes are made. Select and image in the image gallery that has intensities for the image channel you are adjusting.

Note: You will adjust the **Display Intensity** settings on the graph (the Y Axis), the value of the display to (the X axis), the range of pixel intensities. The range of pixel intensities will depend on the instrument and the collection mode set during acquisition. The display range is 0-255; the range of intensities from the camera is 0-4095 for the ImageStream^X or 0-32,767 for EDF mode collection. The IS100 first generation instrument has a 10 bit camera and therefore the range of pixel intensities is 0-1023. The limits of the graph enable you to use the full dynamic range of the display to map the pixel intensities of the image.



At each intensity on the X Axis of the graph, the gray histogram shows the number of pixels in the image. This histogram provides you with a general sense of the range of pixel intensities in the image. The dotted green line maps the pixel intensities to the display intensities, which are in the 0-255 range.

Manual setting is done by Click-dragging the vertical green line on the left side (crossing the X Axis at 0) allows you to set the display pixel intensity to 0 for all intensities that appear to the left of that line. Doing so removes background noise from the image.

Click-dragging the vertical green line on the right side allows you to set the display pixel intensity to 255 for all intensities that appear to the right of that line.

2 From the Image Gallery window, select the object to use for setting the mapping. It appears in the Image Gallery Properties window.

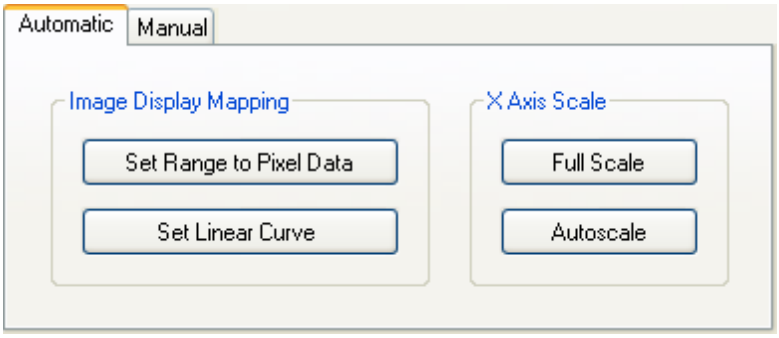
Tip: You might need to select different objects for different channels because an object might not fluoresce in all channels.

3 To adjust the pixel mapping for display, click-drag the vertical green line by clicking near it (but not near the yellow cross).

Tip: For fluorescence channels, set the vertical green line that appears on the left side to the right of the large peak of background pixel intensities as shown above and set the right vertical green line to the right of the brightest pixel intensities. Click 'Set Linear Curve' to make the transformation linear. For the brightfield channel, set the vertical lines to about 50 counts to the right and left of the histogram to produce an image with crisp brightfield contrast.

- To change the mapping curve to be logarithmic or exponential, click-drag the yellow cross.
- To restore the mapping to a linear curve, Click **Set Linear Curve**.
- To see the full scale for the X Axis Click **Full Scale**.
- To set the display mapping of the X Axis to the lowest and highest values for a selected object, Click **Set Range to Pixel Data**.

- To set the scale of the X Axis to the range of the vertical green lines or of all the pixel intensities for the selected object–whichever is larger–Click **Auto-scale**.
- You may enter values manually by selecting the **Manual** tab.



4 If you want to preview the changes in the Image Gallery, click **Preview Changes in Gallery**.

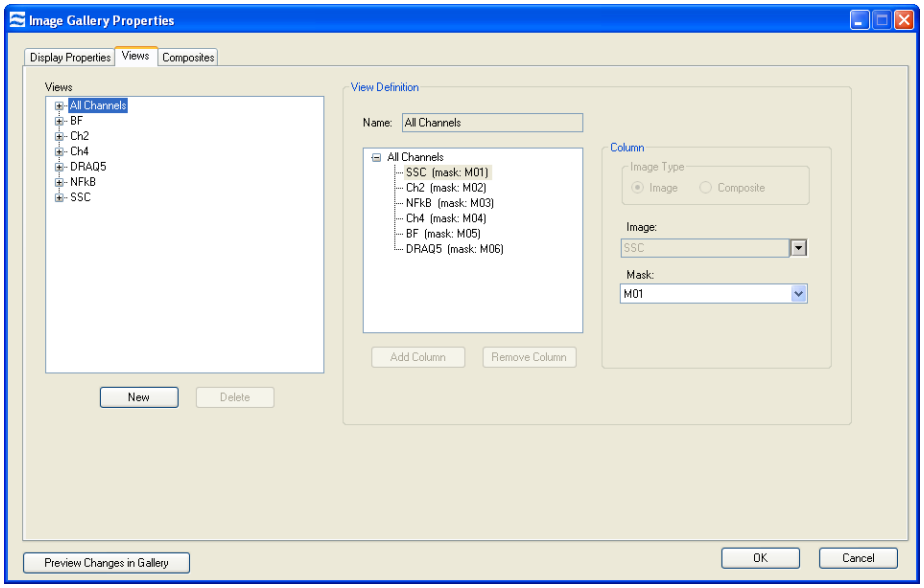
5 Continue customizing the Image Gallery display properties with another procedure in this section, or click **OK** to finish and save changes or **Cancel** to finish and discard changes.

To customize the Image Gallery views images and masks

1 Within the **Image Gallery Properties** window, click the **Views** tab.

Note: The Image Gallery view can be customized to view any combination of channel images or composites. The default view **All Channels** is a view that displays all image channels that were included during acquisition of the file with their associated default masks. This mask may be changed for the default view however, the images in this view cannot be changed.

The list of existing views is shown on the left.



2 To create a new view, Click **New**. The new view is automatically added to the list on the left.

3 In the right section of the window, type in a name for the view.

4 Click **Add Column**.

5 Define the column by selecting an image and a mask or a composite from the dropdown menu.

6 Repeat the previous 2 steps until finished adding columns to the view. A column will be added under the column currently selected. To insert a column click on the image above insertion point.

7 Columns may be removed by clicking on **Remove Column**.

8 A view may be edited at any time by selecting the view and following the same procedures.

9 If you want to delete a view, click the view to select it, and then click **Delete**.

A confirmation window appears.

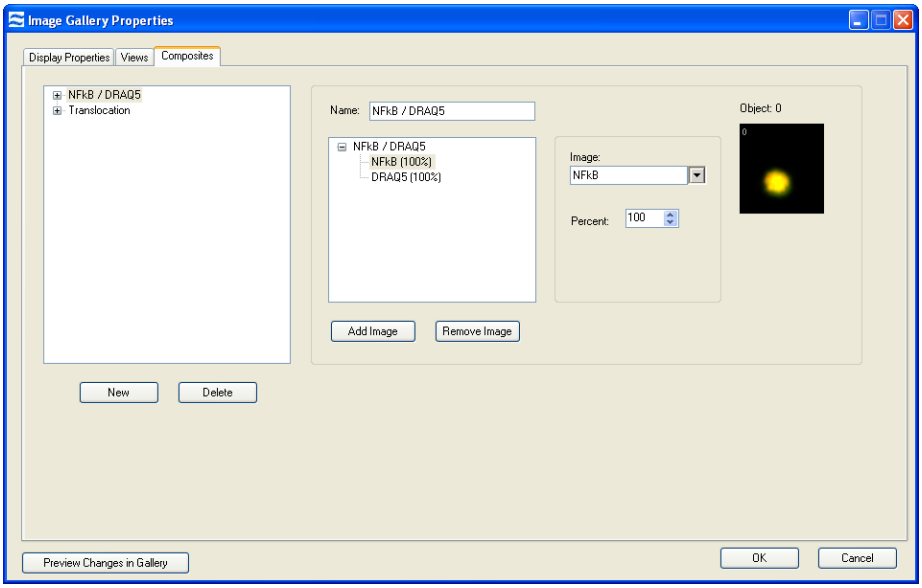
10 If you want to preview any new changes in the Image Gallery, return to the Image Gallery and choose your new view in the **View** drop down menu. Then return to the **Image Gallery Properties** window and click **Preview Changes in Gallery**.

11 Continue customizing the Image Gallery display properties with another procedure in this section, or click **OK** to finish and save changes or **Cancel** to finish and discard changes.

To create a composite

1 Within the **Image Gallery Properties** window, click the **Composites** tab.

The list of existing composites is shown on the left.



2 In the right section of the window, type a name for the composite or leave blank to allow the name to be built automatically from the image names added to the composite.

3 Click **Add Image**. The selected image appears in the **Object** box. Change the **Percent** if desired. The percent specifies the percentage of of the image to include in the composite.

Tip: As you make the changes, the image in the **Object** box updates accordingly. If you want to preview any new changes in the Image Gallery, return to the Image Gallery and select the **View** drop down menu to your new view. Then return to the **Image Gallery Properties** window and click **Preview Changes in Gallery**.

4 Continue to add images as desired.

5 To remove and image from the composite, Click **Remove Image**.

6 The composite is automatically added to the list on the left.

7 A composite can be removed from the list by clicking **Delete**.

8 Continue customizing the Image Gallery display properties with another procedure in this section, or click **OK** to finish and save changes or **Cancel** to finish and discard changes.

Reporting Images and Graphs

The IDEAS application allows users to copy and print images and graphs, export statistics, feature data, pixel data, or TIF files for separate analyses.

Prepare the image gallery and analysis area for reporting.

1 Before you print or copy images, see Setting the Image Gallery Properties to optimize the image display.

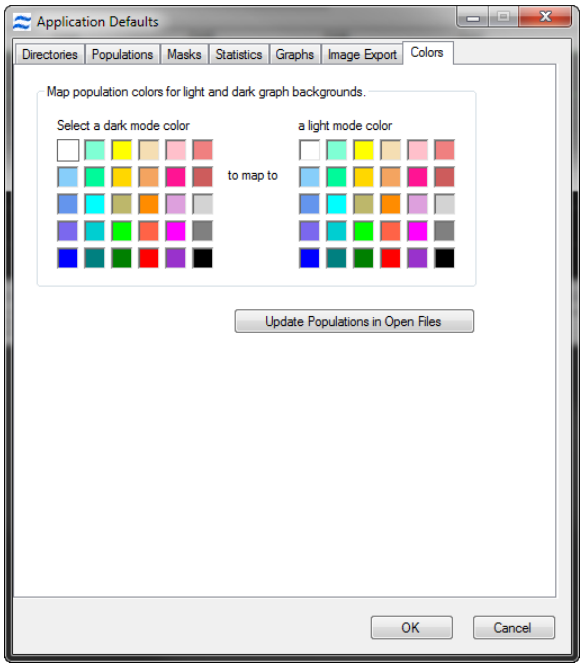
2 In addition to formatting the graphs and statistics in the Analysis area (see Overview of the Analysis Area), the IDEAS application provides color mapping from the dark mode that you see in the Analysis Area to a light mode that has a white background for the printing and exporting of data. Because the population colors might not show on a white background, you can change the colors when using the light mode.

To use light background graphs in the Analysis Area

Click the graph background tool to switch between light or dark mode.

To map the dark mode colors to light mode colors

1 See the Application Defaults-Colors tab .



2 Click **Update Populations in Open Files** when done..

3 Click **OK** to save the changes or **Cancel** to exit.

Copy full or partial screens

To copy the entire screen to the Clipboard

- Press CTRL+PRINT SCREEN. It is then available for pasting into a third party application.

To copy a window to the Clipboard

- Select the window and then press ALT+PRINT SCREEN. It is then available for pasting into a third party application.

Print the analysis area or image gallery directly

To print the Analysis Area data

- Select **Reports** > **Print Analysis Area**.
  The IDEAS application prints all the graphs, statistics, text panels, and images that are displayed in the Analysis Area.

To print the Image Gallery data

- Select **Reports** > **Print Image Gallery**.
  The IDEAS application prints all the images that are visible in the Image Gallery.

Copy images to the clipboard or save images to files

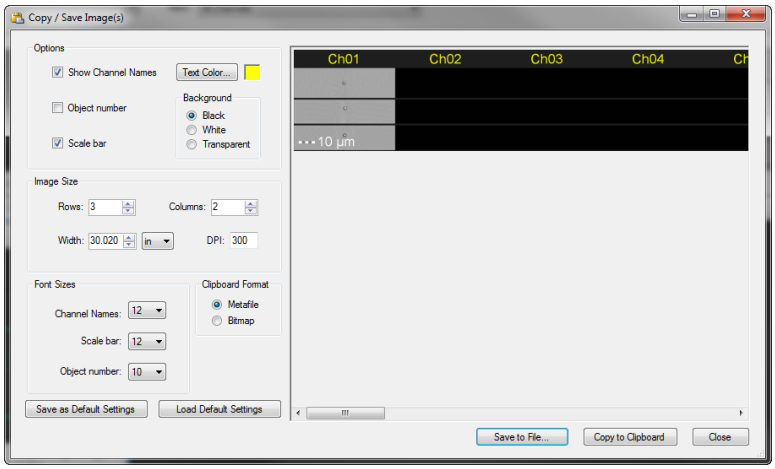
To copy or save Images

1 Right-click anywhere in the Image Gallery and then choose one of the following options:

   - Copy/Save Image for a single image
   - Copy/Save Object Images for the row of images for one object
   - Copy/Save Gallery Column for the images of one channel in the current gallery
   - Copy/Save Gallery for all images in the current gallery

2 A preview of the image(s) is shown. Changes from the default settings may be made to the following:

   - Show Channel names
   - Show Object number
   - Show Scale bar
   - Text color for channel names
   - Background
   - Number of rows and columns
   - Size and DPI settings
   - Font sizes and clipboard format



3 The settings may be Saved to the Default settings or Default settings may be loaded.

4 Click **Save to File** or **Copy to Clipboard** when done. Files may be saved as .png, .bmp or .tif formats.

5 If Copy to Clipboard was chosen, paste into third party application.

Copy graphs to the clipboard or save graphs to a file

To copy or save graphs

1 Right click anywhere in the graph and choose Copy/Save Graph and/or Statistic.

2 A preview of the graph is shown. Changes from the default settings may be made to the following:

- Include Graph
- Graph Size
- Font sizes

These first three settings are loaded from the graph - you may choose to load the default settings that are set in the applications defaults tab.

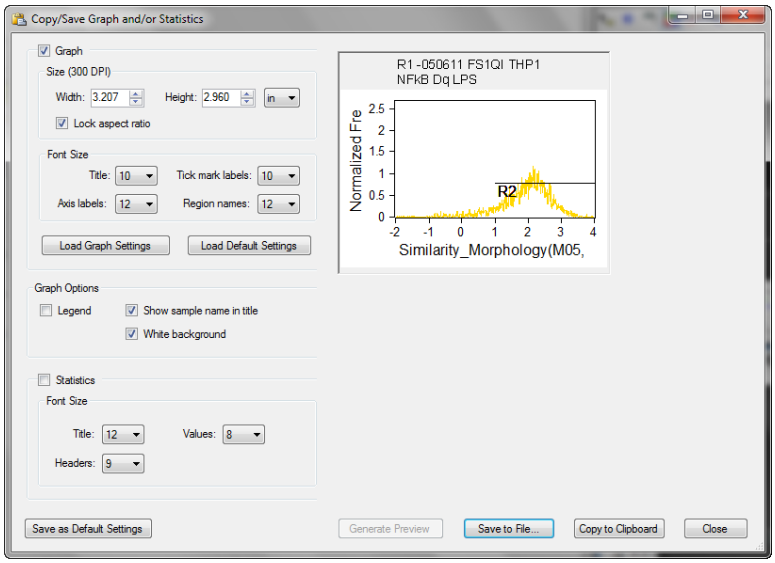
- Include Legend
- Include Sample name in title
- White background on/off
- Include Cursor
- Include Statistics
- Font sizes for statistics

3 If changes are made they can be previewed by clicking **Generate Preview**. Otherwise they will be applied when Save to File or Copy to Clipboard is chosen.

4 Option: settings may be saved to default settings or loaded from default settings.

5 Click **Save to File** or **Copy to Clipboard** when done. Files may be saved as .png, .bmp or .tif formats.

6 If Copy to Clipboard was chosen, paste into third party application.



To export graph statistics to the Clipboard

- Right-click a graph and then click **Export Statistics To Clipboard**. They are then available for pasting into a third party application.

To copy population statistics from a Statistics Table

- Right-click the table and then click **Copy Statistics or Copy Statistics - transposed**. They are then available for pasting into a third party application.

Reporting Statistics

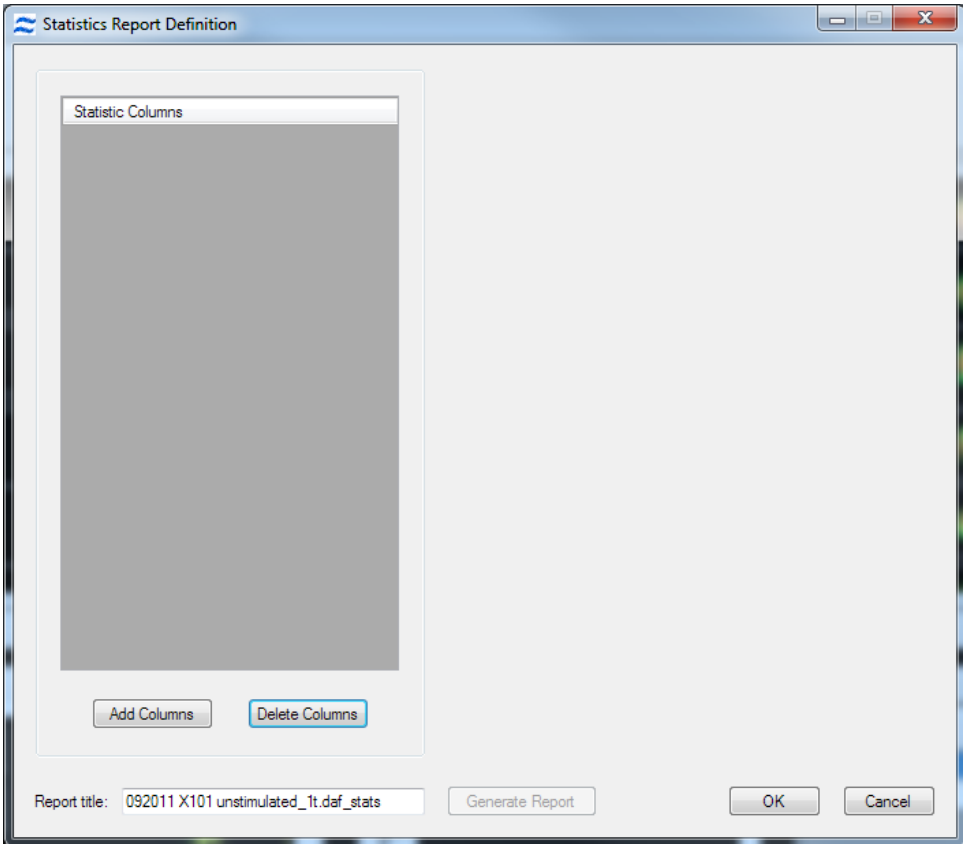
Define a Statistics Report

A statistics report definition can be saved in a .daf file or an .ast template file. It allows users to select specific statistics within a .daf file and open the data in Excel.

A statistics report can be generated during batching if it is part of the template used. It may also be applied to pre-existing .daf files from the Reporting menu. In this case, the rest of the template is not processed–only the report. The statistics report definition allows you to specify population percentages and feature statistics and the layout of the report is accessed from the reporting menu.

To create a Statistics Report Definition

1 Select **Reports** > **Define Statistics Report**.
The Statistics Report Definition appears.



2 Enter a **Report title**.

3 Under the **Statistic Columns** click**Add Columns**. The Add Statistic Report Column window opens.

4 Select the statistic(s) in the **Statistics** list.

- **Count** - the absolute count of the populations
- **%Total** - percentage of a population as a percentage of All

- **%Gated** - the percent of one population as a percentage of another, but not used for tagged populations
- **%** - the percentage of one population as a percentage of another, also is used for tagged populations
- **Objects/mL** - the concentration of the population in the sample run.
- **CV** - the coefficient variable
- **Geometric Mean** - standard statistical definition
- **Maximum** - standard statistical definition
- **Mean** - standard statistical definition
- **Median** - standard statistical definition
- **MAD** - standard statistical definition
- **Minimum** - standard statistical definition
- **Mode** - standard statistical definition
- **RD - Median** - the ratio discriminant (Fisher's discriminant) using the Median and MAD
- **RD - Mean** - the ratio discriminant (Fisher's discriminant) using the Mean and StdDev
- **Standard Deviation** - standard statistical definition
- **Variance** - standard statistical definition
- **NaN** - stands for not a number; the number of objects whose values are not valid numbers.

5 Select a population to base on the selected statistic(s).

6 Select a reference population if necessary. This is required for % and RD.

7 Select a **Feature**. This is not necessary for the %-related statistics, Count or Objects/ml.

8 Click **Add Statistics**.The statistic is added to the list.

9 Click **Close** when finished.

10 Select a statistic in the list to view the definition or edit any input.

11 Change the name of the statistic by unchecking **Use default title** and typing a new name if desired..

12 **Delete Columns** removes a selected statistic.

13 To reorder the list click-drag a statistic to it's new location.

14 Click **Generate Report** when complete to generate a report for a current (opened) .daf file.

A prompt appears to save the text file. This text file can be opened from Excel.

15 If you do not want to generate a report, click **OK** to save your changes and exit the window.

16 The saved template can generate a statistics report for multiple data files by selecting Generate Statistics Report from the Reports menu or during batch processing.

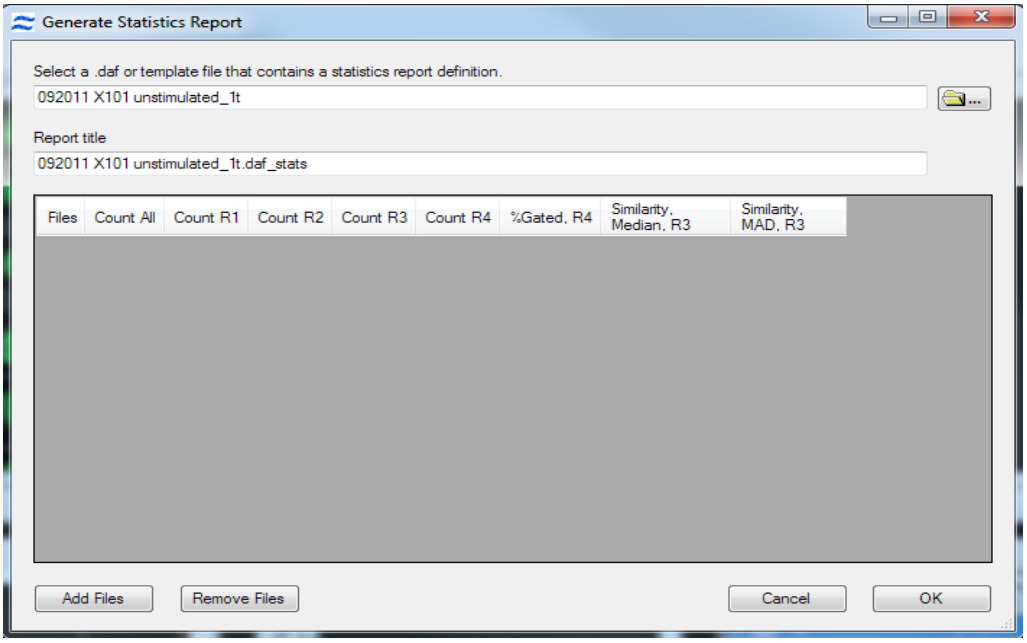
Generating a Statistics Report using .daf Files

Once a Statistics Definition has been created, the user can generate a statistics report from multiple .daf files. However, these files must use the same template. Batch Processing can also generate a statistics report where statistics for each data file will be generated either for .rif, .cif, or .daf files. Generating a statistics report under the Reports menu simply adds the statistics template to the specified .daf files.

To Generate a Statistics Report

1 Select **Reports** > **Generate Statistics Report**.

The current .daf file appears in the window with the specified statistics columns.



2 Pick a **Report Definition**. The definition may be obtained from a .daf or .ast file.

3 Change the **Report title** if desired.

4 Additional .daf files can be added or removed with the **Add Files** or **Remove Files** buttons.

5 Reorder the files as desired by selecting files and then right-click the new location in the list and choose move here. You can Ctrl select multiple files in the desired order and then move all at once by right-clicking in the desired location and choosing move here.

6 Click **OK**.

A prompt will confirm that the .daf file will be saved. The report title name will be used as the default file name for the report. In the above example, the file generated will be named “Report 1.txt”. If the report title contains illegal characters, such as “\/><“ the default filename will change to “Statistics Report.txt”. Tab delimited text format is used for the report.

Reporting Statistics from a Single Graph or Statistics Table

Statistics can also be reported directly from an open .daf from the graph or statistics tables in the analysis area.

To export graph statistics to the Clipboard

- Right-click a graph and then click **Export Statistics To Clipboard**. They are then available for pasting into a third party application.

To copy population statistics from a Statistics Table

- Right-click the table and then click **Copy Statistics or Copy Statistics - transposed**. They are then available for pasting into a third party application.

Exporting Data

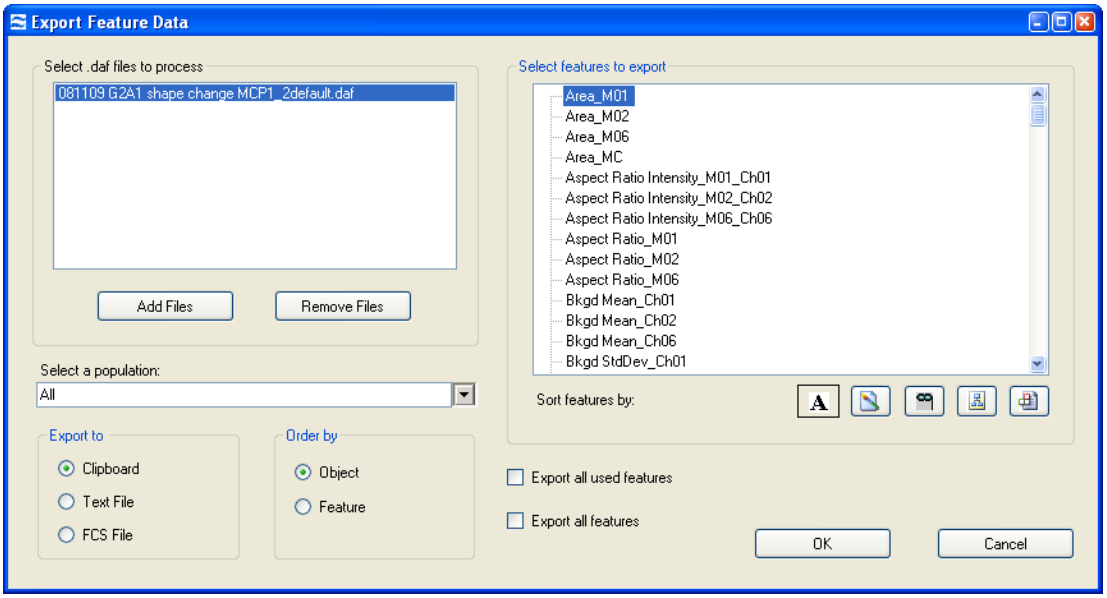
You can export feature values for a population to the Clipboard, a text file, or a Flow Cytometry Standard (FCS) file. You can export pixel intensity values for an object to the Clipboard or a text file. Later, you can open or paste the FCS file into a spreadsheet or other programs that uses the FCS file format. Keep in mind, however, that limitations might exist on the number of values that these programs can import.

Exporting Feature Data

Exporting feature data is useful if you want to create an fcs file or graph the feature data in a third party graphing application.

To export feature data

1 On the **Tools** menu, click **Export Feature Values**.
The **Export Feature Data** window appears.



2 Add files to the list on the left to export values for multiple files.

3 In the **Select a population** drop down menu, select the population that you want.
If you haven't defined any populations, **All** is the default. To make a new population, refer to Creating Tagged Populations.

4 In the **Select feature values to export** area, select features by clicking items in the list or hold down the Ctrl while clicking to select multiple items.

5 Select the **Export to** option that you want. Note that data exported to the Clipboard can be pasted directly into a spreadsheet program.

6 Select the **Order by** option that you want. Note that ordering by object causes the values to be listed in a column, whereas ordering by feature causes the values to be listed in a row.

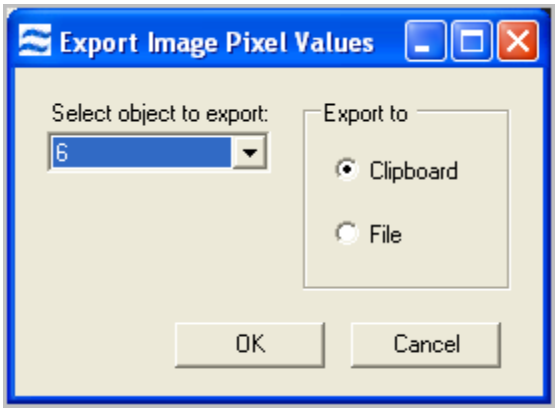
7 Click **OK**.

Exporting Pixel Data

Exporting pixel data is useful when importing the data into third-party programs where you would need to graph the individual pixels.

To export pixel data

1 On the **Tools** menu, click **Export Image Pixel Values**.
The **Export Image Pixel Values** window appears.



2 **Select the object to export** in the drop down menu.
3 Select to **Export to** either the **Clipboard** or **File**.
4 Click **OK**.
5 Paste into desired application.

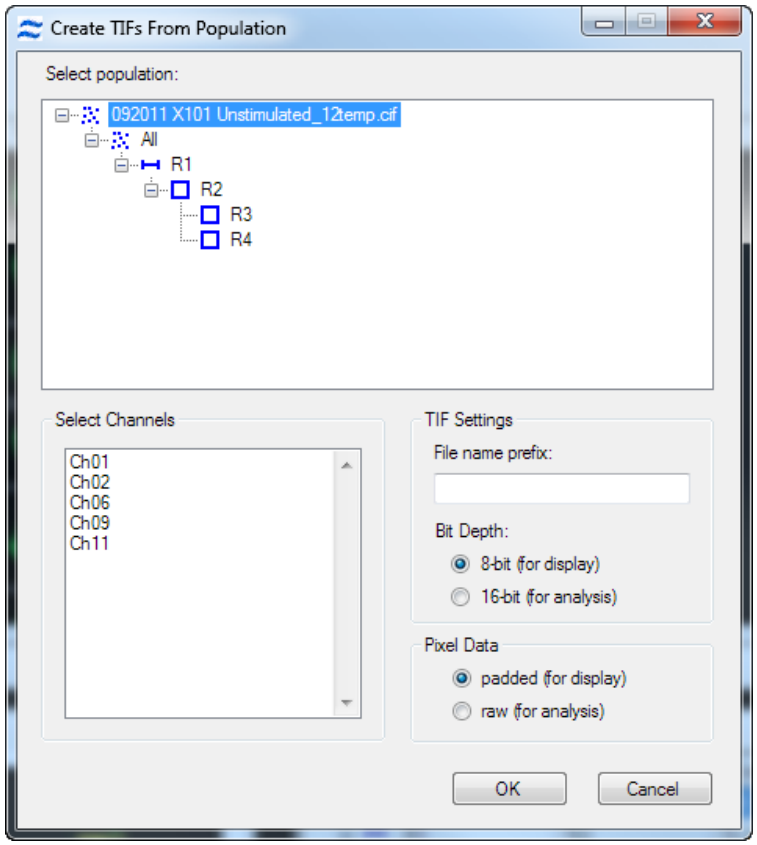
Creating TIFs From Population for Export

The IDEAS application allows users to create separate TIF files for channel images for every event in that population. The exported TIF files can be opened in image viewing applications that support 8 bit tif format for display or 16 bit tif format for analysis.

To create TIFs From Population for Export

1 On the **Tools** menu, click **Export .tif Images**.

The Create TIFs From Population window appears.



2 Select the population and channels.

3 Type a prefix for the TIF file name.

4 Select the bit depth.

5 Select padded or raw.

6 Click **OK**.

A TIF file is created for every selected channel within the selected population.

Printing Data

The IDEAS application provides color mapping from the dark mode that you see in the Analysis Area to a light mode that has a white background for the printing and exporting of data. Because the population colors might not show on a white background, you can change the colors when using the light mode.See Application Defaults for more information.

To use light background graphs in the Analysis Area

- Click the graph background tool to switch between light or dark mode.
- See Viewing and Changing the Application Defaults - colors tab to set the color mapping desired.

To print the Analysis Area data

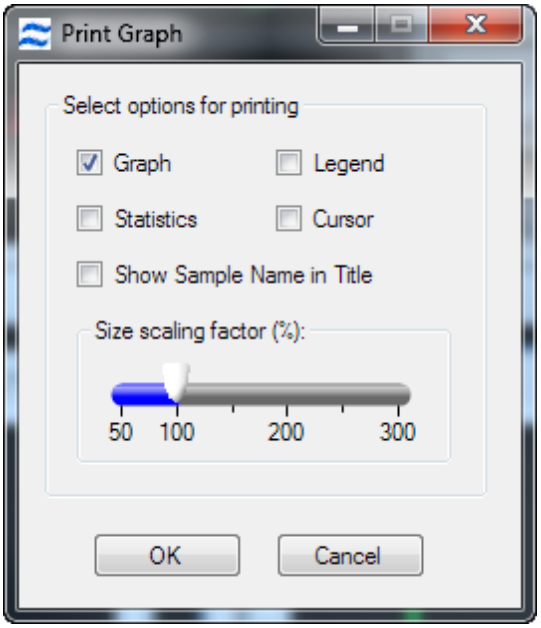
- Select **Reports** > **Print Analysis Area**.
  The IDEAS application prints all the graphs, statistics, text panels, and images that are displayed in the Analysis Area.

To print the Image Gallery data

- Select **Reports** > **Print Image Gallery**.
  The IDEAS application prints all the images that are visible in the Image Gallery.

To print an individual graph

1 Right-click the graph and then select **Print Graph** on the graph context menu. The Print Graph window appears.

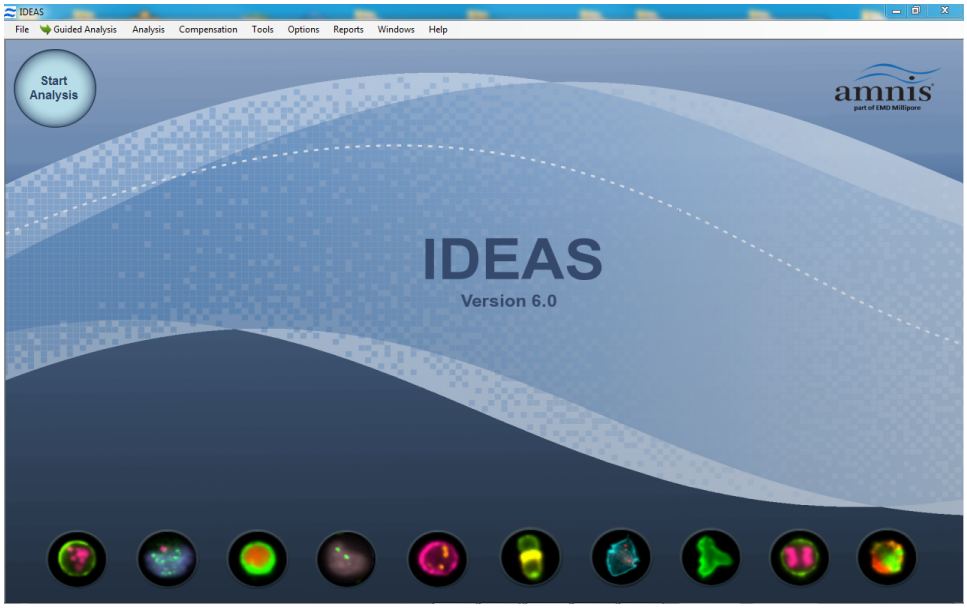


2 Select the checkboxes **Graph**, **Statistics**, **Legend**, **Cursor, Show Sample Name in Title** to include the elements in the report.

3 If necessary, adjust the size scaling factor. Recomended setting is 100%.

4 Click **OK** to print the graph.

Chapter 4: Features and Masks



Understanding the IDEAS® Features and Masks

This section contains the following subsections, which describe the features that the IDEAS application uses for data analysis:

See the following lists of base features:

Table of Base Features - Alphabetical

Table of Base Features- by Category

Table of Basic Features available for FlowSight without QI

Understanding the Size Features

Understanding the Location Features

Understanding the Shape Features

Understanding the Texture Features

Understanding the Signal Strength Features

Understanding the System Features

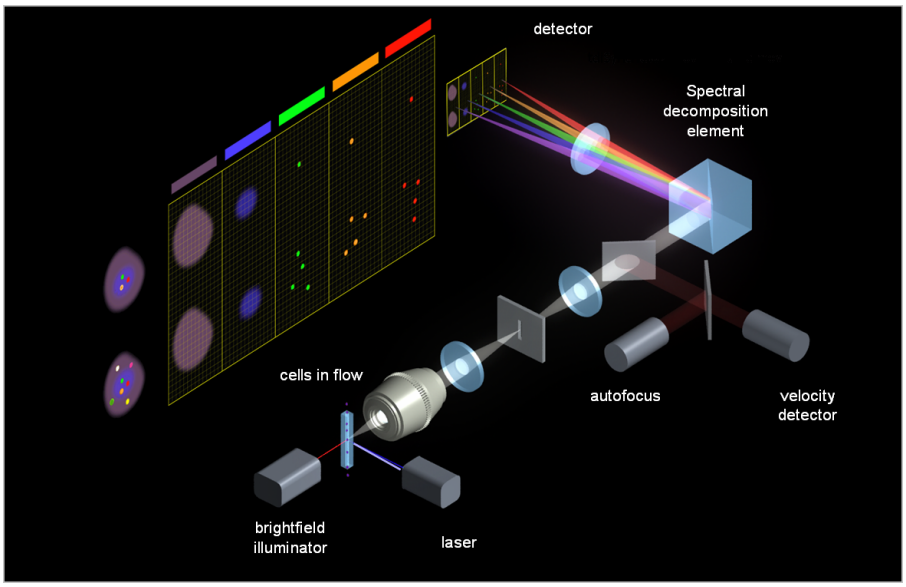
Understanding the Comparison Features

About Masks

Mask Functions

Overview of the IDEAS Features and Masks

Objects passing through an Amnis cell analysis system are illuminated in different directions by lasers and/or brightfield LEDs. Light emitted from the object is focused through an objective lens and relayed to a spectral decomposition element, which divides the light into six spectral bands located side-by-side across a charge-coupled detector (CCD), as shown in the following diagram. Therefore, each object has six images that can be individually analyzed or, because they are in spatial register with respect to one another, reconstructed. Each of the separate bands is called a channel. Below is an example of collecting 6 images. The ImageStreamx system has a second camera option which enables collection of up to 12 images per object. The FlowSight system has 12 channels collection on 1 camera.



About Features

The IDEAS application provides a large selection of criteria, or features, for analyzing images. A feature is described by a mathematical expression that contains quantitative and positional information about the image. A feature is applied to specific locations of an image by the use of a mask that identifies pixels within the region of interest of the image. A few system features, such as Object Number, Camera Background and Flow Speed, do not require calculations, masks, or image intensity information.

There is a slight difference in features created during data acquisition and those in IDEAS. During acquisition features are created with the INSPIRE mask. Features and masks are calculated in IDEAS for files collected with the ImageStream or a FlowSight with the Quantitative Imaging (QI) upgrade.

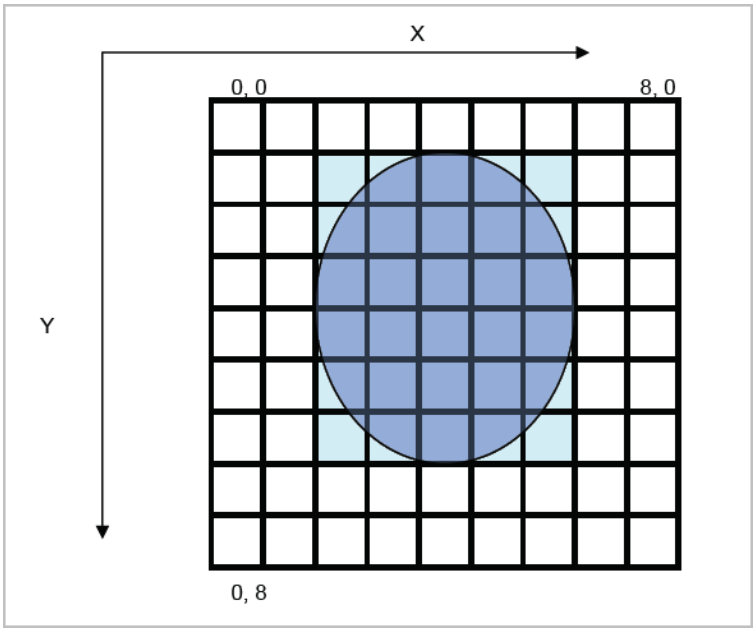
New masks and features can be created in files from an ImageStream or FlowSight with the QI upgrade using the Mask and Feature Manager tools. Features are

created in IDEAS using base feature algorithms,such as Area or Intensity along with a mask and/or a channel image for files created with an ImageStream or with the QI upgraded FlowSight machine. The default masks are recomputed in IDEAS for ImageStream or QI enabled files. Combined features can be created using existing features in mathematical expressions in the Feature Manager.

IDEAS groups the features into eight categories: size, location, shape, texture, signal strength, comparison, system and combined.

For more information, see Overview of the Mask Manager

To calculate the value of a feature, the IDEAS application maps the channel image to X and Y coordinates, as illustrated by the following diagram. Each box in the diagram represents a pixel.



The pixel size and field of view per channel is dependent on the magnification used. See your INSPIRE Users Manual for more information.

Features Categories

Size

Size features are in microns and include Area, Diameter, Length, Major Axis, Minor Axis, Major Axis Intensity, Mnor Axis Intensity, Perimeter, Thickness Max and Min, Spot Area Min, Width and Height.

Location

Location features include Angle, Angle Intensity, Centroid X, Centroid Y, Centroid X Intensity, Centroid Y Intensity, Delta Centroid X, Delta Centroid Y, Delta Centroid XY, Max Contour position, Spot Distance Min, Valley X and Valley Y.

Shape

Shape features define the mask shape and have units that vary with the feature. They include the Aspect Ratio, Aspect Ratio Intensity, Compactness, Elongatedness, Lobe Count, and Symmetry 2,3,4.

Texture

The Texture features determine local intensity variations in images and include Bright Detail Intensity R3 and Bright Detail Intensity R7, Contrast, Gradient Max, Gradient RMS, H Texture (H-Contrast, H-Correlation, H-Energy, H-Entropy, H-Homogeneity, and H-Variance), Modulation, Spot Count, and Std Dev.

Contrast, Gradient Max and Gradient RMS are generally used to determine best focus.

Comparison

The Comparison features describe the difference of intensity measurements between masks or pixels in different images or the same image with different masks. These include Bright Detail Similarity R3, Intensity Concentration Ratio, Internalization, and Similarity.

System

The system features do not require a mask.

Table of Base Features - Alphabetical

Delete this text and replace it with your own content.

Feature Name	Category
Angle Feature	Location
Angle Feature	Location
Area Feature	Size
Aspect Ratio Feature	Shape
Aspect Ratio Intensity Feature	Shape
Bkgd Mean Feature	Signal Strength
Bkgd StdDev Feature	Signal Strength
Bright Detail Intensity R3 and Bright detail Intensity R7 Features	Signal Strength
Bright Detail Similarity R3 Feature	Comparison
Camera Line Number Feature	System
Camera Timer Feature	System
Centroid Features	Location
Centroid Features	Location
Circularity Feature	Shape
	Shape
Contrast Feature	Texture
Centroid Features	Location
Centroid Features	Location
Diameter Feature	Size
Elongatedness Feature	Shape
Elongatedness Feature	Texture
Flow Speed Feature	System
Gradient Max Feature	Texture
Gradient RMS Feature	Texture
Height Feature	Size
H Texture Features	Texture
Intensity Concentration Ratio Feature	Comparison
Intensity Feature	Signal Strength
Internalization Feature	Comparison
Length Feature	Size
Lobe Count Feature	Shape
Major Axis and Minor Axis Features	Size
Major Axis Intensity and Minor Axis Intensity Features	Size
Max Contour Position Feature	Location
Max Pixel Feature	Signal Strength

Feature Name	Category
Mean Pixel Feature	Signal Strength
Median Pixel Feature	Signal Strength
Min Pixel Feature	Signal Strength
Minor Axis: see Major Axis Intensity and Minor Axis Intensity Features	Size
Modulation Feature	Texture
Object Number Feature	System
Objects/ml Feature	System
Objects/sec Feature	System
Perimeter Feature	Size
Spot Intensity Min and Spot Intensity Max Features	Location
Raw Intensity Feature	Signal Strength
Raw Max Pixel Feature	Signal Strength
Raw Mean Pixel Feature	Signal Strength
Raw Min Pixel Feature	Signal Strength
Raw Median Pixel Feature	Signal Strength
Saturation Count Feature	Signal Strength
Saturation Percent Features	Signal Strength
Shape Ratio Feature	Shape
Similarity Feature	Comparison
Similarity Feature	Comparison
Spot Area Min Feature	Size
Spot Count Feature	Texture
Spot Distance Min Feature	Location
Spot Intensity Min and Spot Intensity Max Features	Signal Strength
Std Dev Feature	Texture
Symmetry 2, 3, 4 Features	Texture
Thickness Max Feature	Size
Table of Base Features- by Category	Size
Time Feature	System
Valley X and Valley Y Features	Location
Width Feature	Size
XCorr Feature	Comparison

Table of Base Features- by Category

Table 1: List of Features by category

Feature category	Feature name	In Default Template?	In Expanded Default Template?	Mask_Image Used in Default Template
Size	**Size-based Features are in microns.**			
	Area Feature The size of the mask in square microns.	Yes	Yes	M01-M12, MC
	Diameter Feature Estimates the diameter of the mask based on Area.	No	Yes	M01-M12
	Height Feature Based on a bounding rectangle, the Width is the smaller side and the Height is the longer side of the rectangle.	Yes	Yes	M01-M12
	Length Feature Measures the longest part of the mask.	Yes	Yes	M01-M12
	Major Axis and Minor Axis Features Describes the widest part of the mask and the narrowest part of the mask, respectively.	No	Yes	M01-M12
	Major Axis Intensity and Minor Axis Intensity Features Based on a bounding ellipse, the Minor Axis is the narrow part and the Major Axis is the widest part.	No	Yes	M01_Ch01-M12_Ch12
	Minor Axis:Major Axis and Minor Axis Features	No	Yes	M01-M12
	Perimeter Feature Describes circumference of the mask.	No	Yes	M01-M12
	Spot Area Min Feature The Area of the smallest spot in the mask. (See also Spot Distance Min Feature, Spot Intensity Min and Spot Intensity Max Features and Spot Count Feature.	No	No	
	Thickness Max Feature Describes the longest width of the mask.	No	Yes	M01-M12
	Thickness Min Feature Describes the shortest width of the mask.	No	Yes	M01-M12
	Width Feature Based on a bounding rectangle, the Width is the smaller side and the Height is the longer side of the rectangle.	Yes	Yes	M01-M12
Location	**Location Features are in X,Y pixel coordinates from an origin in the upper left corner, pixels or contour**			
	Angle Feature The angle of the major axis from a horizontal plane in radians.	No	No	
	Angle Feature The angle of the major axis intensity from a horizontal plane in radians.	No	No	
	Centroid Features The central tendency of the pixels along the X Axis and	No	Yes	M01-M12

Feature category	Feature name	In Default Template?	In Expanded Default Template?	Mask_Image Used in Default Template
	Y Axis, respectively.			
	Centroid Features The central tendency of the pixels along the X Axis and Y Axis, respectively, with the pixel intensities weighted.	No	Yes	M01_Ch01-M12_Ch12
	Centroid Features The distance between the X or Y Centroids of two images.	No	No	
	Centroid Features The distance between the Centroids of two images.	No	No	
	Max Contour Position Feature The location of the contour in the cell that has the highest intensity concentration.	No	No	
	Spot Distance Min Feature The shortest distance between two components (spots). See also Spot Area Min Feature, Spot Intensity Min and Spot Intensity Max Features and Spot Count Feature.	Yes	No	
	Valley X and Valley Y Features The (X,Y) coordinates of the minimum intensity within the skeletal lines that are used when creating the Valley Mask.	No	No	
Shape	**Shape Features define the mask shape and have units that vary with the feature.**			
	Aspect Ratio Feature The ratio of the Minor Axis divided by the Major Axis.	Yes	Yes	M01-M12
	Aspect Ratio Intensity Feature The ratio of the Minor Axis Intensity divided by the Major Axis Intensity.	Yes	Yes	M01_Ch01-M12_Ch12
	Circularity Feature The degree of the mask's deviation from a circle.	No	No	
	Describes the density of intensities within the object.	No	No	
	Elongatedness Feature The ratio of the Height/Width which use the bounding box.	No	Yes	M01-M12
	Lobe Count Feature The number of lobes in a cell. (Also see Symmetry)	No	No	
	Shape Ratio Feature The ratio of Thickness Min/Length features.	No	Yes	M01-M12
	Symmetry 2, 3, 4 Features These three features measure the tendency of the object to have a single axis of elongation, a three-fold and a four-fold variation of the shapes. See also Lobe Count Feature.	No	No	
Texture	**Texture features measure pixel or regional variation and indicate the granularity or complexity of the**			

Feature category	Feature name	In Default Template?	In Expanded Default Template?	Mask_Image Used in Default Template
	image.			
	Bright Detail Intensity R3 and Bright detail Intensity R7 Features The Intensity of the pixels in the bright detail image using a 3 or 7 pixel structuring element. Also, see Spot Mask for a description of the bright detail image.	Yes (R3) No (R7)	Yes	MC_Ch1-MC_Ch6
	Contrast Feature Enumerates changes of pixel values in the image to measure the focus quality of an image.	Yes	Yes	M01_Ch01-M12_Ch12
	Gradient Max Feature The maximum slope of the pixel value changes in the image to measure focus quality of an image.	No	Yes	M01_Ch01-M12_Ch12
	Gradient RMS Feature Enumerates changes of pixel values in the image to measure the focus quality of an image.	Yes	Yes	M01_Ch01-M12_Ch12
	H Texture Features Measures Haralick texture features.	No	Yes	M01_Ch01_5-M12_Ch12_5
	Modulation Feature Measures the intensity range of an image, normalized between 0 and 1.	Yes	Yes	M01_Ch01-M12_Ch12
	Spot Count Feature Enumerates the number of spots. See also Spot Distance Min Feature, Spot Area Min Feature, and Spot Intensity Min and Spot Intensity Max Features.	No	No	
	Std Dev Feature Describes the overall distribution of pixel intensities.	No	Yes	M01_Ch01-M12_Ch12
Signal Strength	**Signal Strength Features are measured in pixel values.**			
	Bkgd Mean Feature The average intensity of the camera background.	Yes	Yes	Ch01-Ch12
	Bkgd StdDev Feature The standard deviation of the background intensities.	No	Yes	Ch01-Ch12
	Intensity Feature The sum of the pixel intensities in the mask, background subtracted.	Yes	Yes	MC_Ch01-MC_Ch12
	Max Pixel Feature The largest pixel value within the mask, background subtracted.	Yes	Yes	MC_Ch01-MC_Ch12
	Mean Pixel Feature The average pixel value within the mask, background subtracted.	Yes	Yes	M01_Ch01-M12_Ch12
	Median Pixel Feature The median pixel value within the mask, background sub-	Yes	Yes	M01_Ch01-M12_Ch12

Feature category	Feature name	In Default Template?	In Expanded Default Template?	Mask_Image Used in Default Tem-plate
	tracted.			
	Min Pixel Feature The smallest pixel value within the mask, background subtracted.	No	No	
	Raw Intensity Feature The sum of the pixel intensities within the mask.	No	No	
	Raw Max Pixel Feature The largest pixel intensity.	Yes	Yes	MC_Ch01-MC_Ch12
	Raw Mean Pixel Feature The average pixel intensity: Raw does not have back-ground subtracted.	No	No	
	Raw Median Pixel Feature The median pixel intensity.	No	No	
	Raw Min Pixel Feature The lowest pixel value within the mask.	Yes	Yes	MC_Ch01-MC_Ch12
	Saturation Count Feature The number of pixels in the mask that are saturated.	Yes	Yes	M01_Ch01-M12_Ch12
	Saturation Percent Features The Percentage of pixels in the mask that are saturated.	Yes	Yes	
	Spot Intensity Min and Spot Intensity Max Features The raw intensity (not background subtracted) of the dim-mest component (spot). See also Spot Count Feature, Spot Distance Min Fea-ture, and Spot Area Min Feature.	No	No	
Com-parison	**Difference of intensity measurements between masks or pixels.**			
	Bright Detail Similarity R3 Feature Measures the correlation of the bright details between image pairs.	No	No	
	Intensity Concentration Ratio Feature Given two masks, the ratio of the intensity in one mask to the total intensity in both masks.	No	No	
	Internalization Feature The ratio of the intensity inside the cell to the intensity of the entire cell.	No	No	
	Similarity Feature The Similarity is a measure of the degree to which two images are linearly correlated pixel by pixel within a masked region.	No	No	
	XCorr Feature The XCorr is a measure of the degree to which two images frequencies are cross correlated.	No	No	
System	**System features do not require a mask and tend to deal with system wide metrics.**			
	Camera Line Number Feature	No	Yes	

Feature category	Feature name	In Default Template?	In Expanded Default Template?	Mask_Image Used in Default Template
	An incremental count of objects.			
	Camera Timer Feature The clock rate in KHz. This is relative time.	No	Yes	
	Flow Speed Feature The calculated flow speed in mm/sec.	Yes	Yes	
	Object Number Feature The sequence of objects.	Yes	Yes	
	Objects/ml Feature A local concentration of all objects per ml. Note: to get objects per ml of a population, use the statistic 'Concentration'.	No	Yes	
	Objects/sec Feature A local concentration of number of objects per second. Note: to get objects per ml of a population, use the statistic 'Concentration'	No	Yes	
	Time Feature The camera timer feature, converted to seconds.	Yes	Yes	
Combined	**Any combined feature will be listed under Combined**	No	No	

Table of Basic Features available for FlowSight without QI

The default masks used for FlowSight Basic (non-QI) files is the INSPIRE mask.

FlowSight Basic Features

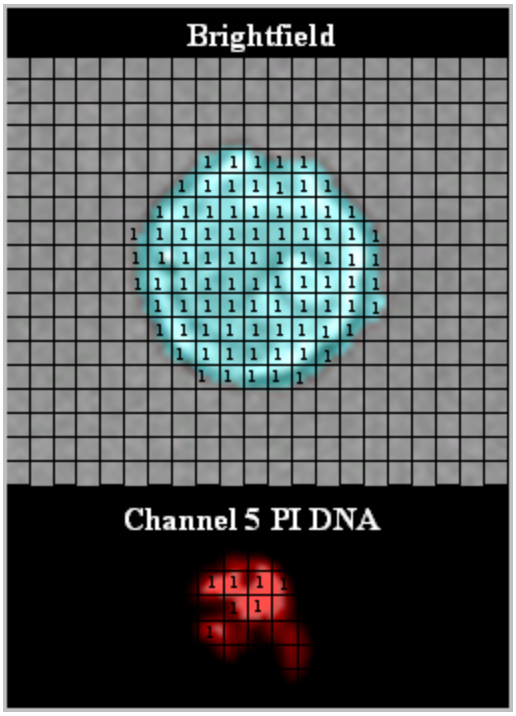
Feature	Mask_Channel	Brief definition
Area	M01-GM, MC	The size of the mask in square microns.
Aspect Ratio	M01-M12	The ratio of the Minor Axis divided by the Major Axis.
Bkgd Mean	Ch01-Ch12	The average intensity of the camera background.
Bkgd StdDev	Ch01-Ch12	The standard deviation of the background intensities.
Camera Line Number	none	An incremental count of objects.
Gradient RMS	M01_Ch01-M12_Ch12	Enumerates changes of pixel values in the image to measure the focus quality of an image.
Intensity	MC_Ch01 - MC_Ch12	The sum of the pixel intensities in the mask, background subtracted.
Minor Axis	M01-M12	Describes the narrowest part of the mask.
Object Number	none	The sequence of objects.
Raw Centroid X Raw Centroid Y	none	The central tendency of the pixels along the X Axis and Y axis respectively
Raw Max Pixel	MC_Ch01- MC_Ch12	The largest pixel value.
Raw Min Pixel	MC_Ch01 - MC_Ch12	The lowest pixel value.
Uncompensated Intensity	MC_Ch01- MC_Ch12	The sum of the pixel intensities in the mask, background subtracted, no compensation applied.

Understanding the Size Features

Size features are in microns and include Area, Diameter, Length, Major Axis, Minor Asix, Major Axis Intensity, Minor Axis Intensity, Perimeter, Thickness Max and Min, Spot Area Min, Width and Height.

Area Feature

The number of microns squared in a mask is equal to the Area. In the following figure, a 1 symbolizes whether the area is included in the mask. The number of pixels is converted to μm^2. Note that 1 pixel = 0.25 μm^2. As an example, a cell with a mask that includes 2000 pixels is therefore equal to 500 μm^2.



Application Examples:

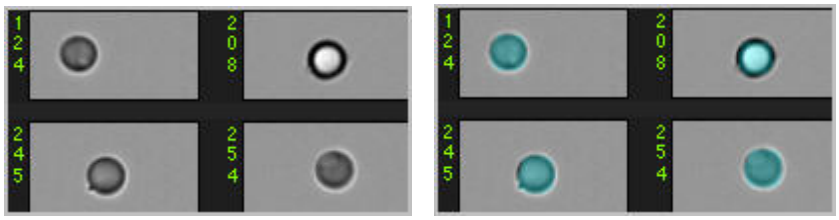
- Quantify and compare cell size.
- Identify single cells.
- Calculate the radius, diameter and volume of the cell.
- Identify apoptosis using the Area of the 30% threshold mask of a nuclear dye.
- Create a pseudo FSC va. SSC plot for comparing with flow cytometry.

Diameter Feature

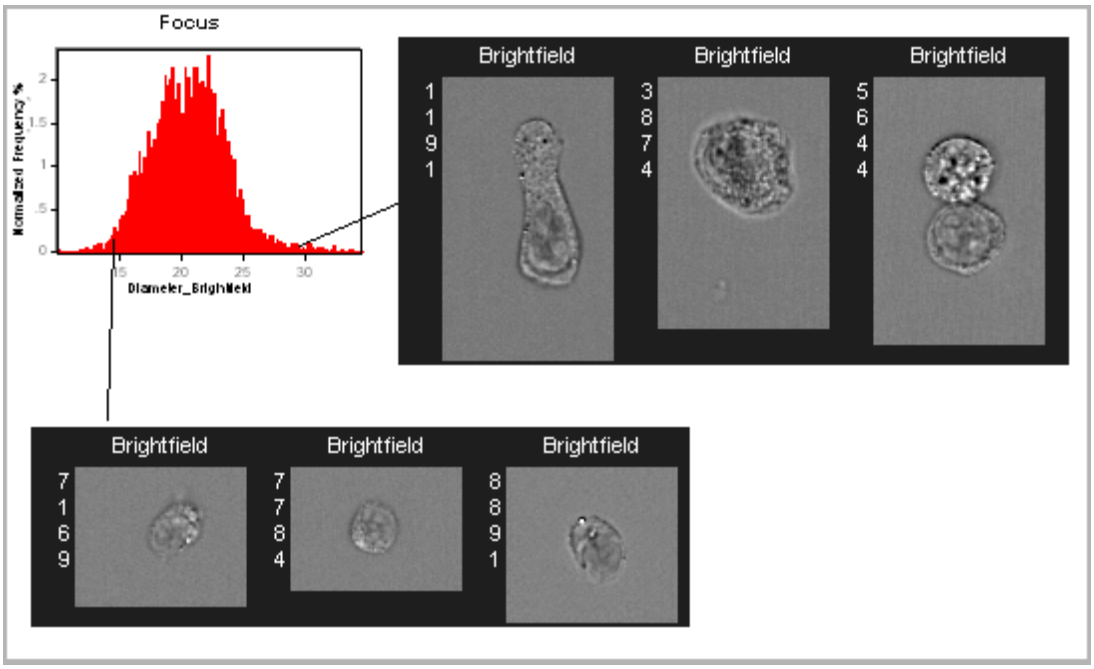
The Diameter feature provides the diameter of the circle that has the same area as the object. The accuracy of the diameter is highly dependent on a close fitting mask and roundness of the cell.

$$Diameter = 2 \times \sqrt{\frac{Area}{\pi}}$$

The images below depicts beads with a uniform diameter of 9 microns.



In the next figure, note that images with longer shapes that have the same area will have the same diameter value.

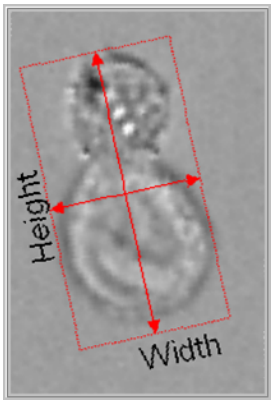


Application Example:

– Used to obtain approximate size of the cell.

Height Feature

Using the bounding rectangle, Height is the number of microns of the longer side and Width the shorter side. See also Elongatedness Feature.



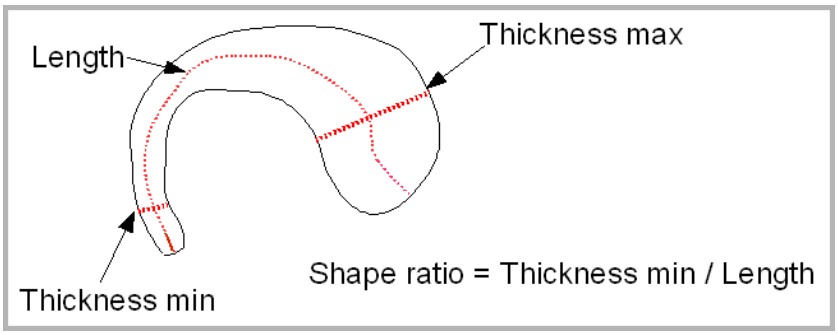
Application Example:

– These features can be used to separate rectangular shaped objects. For curved objects, measurement is more accurately obtained using the thickness features.

Length Feature

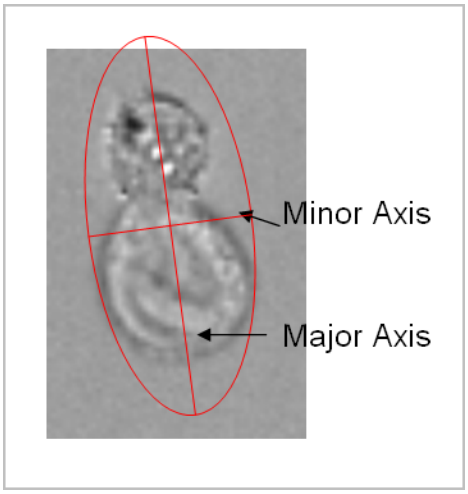
Length measures the longest part of an object. Unlike the Major Axis feature, Length can measure the object's length even if it folds to form a cashew, banana, or doughnut shape, where in many of these cases the major or minor axis features would not be able to differentiate these with true circular shaped objects with no hole.

This feature is based on an input mask and is sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important. See the Shape Ratio Feature, Table of Base Features- by Category , and Thickness Max Feature for more information.



Major Axis and Minor Axis Features

The Major Axis is the longest dimension of an ellipse of best fit. The Minor Axis is the narrowest dimension of the ellipse of best fit. See the Aspect Ratio Feature for more information.

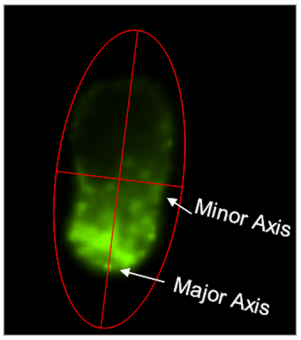


Application Examples:

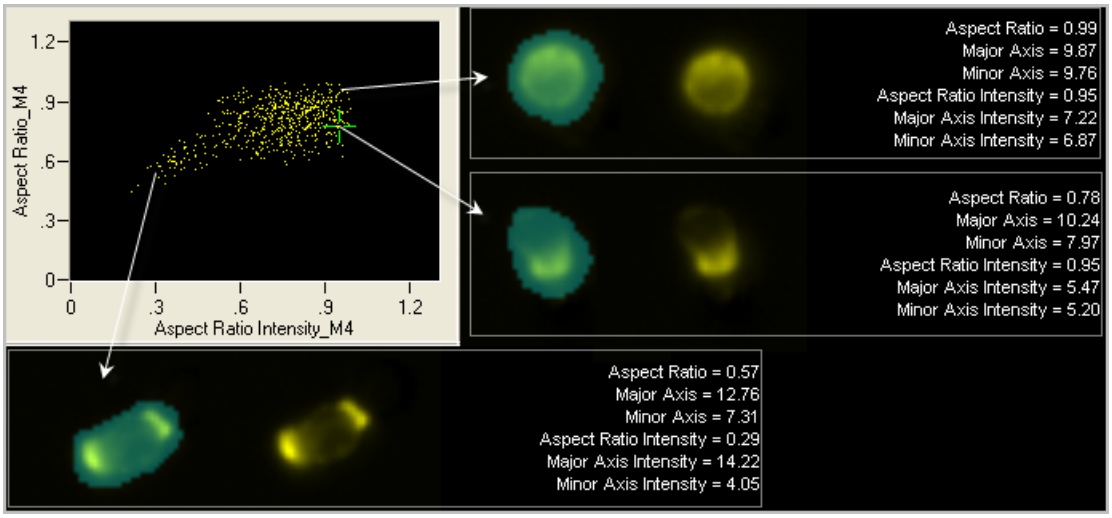
- Quantify and compare cell shape.
- Identify small, medium, and large cells.

Major Axis Intensity and Minor Axis Intensity Features

The Major Axis Intensity is the longest dimension of an ellipse of best fit and is intensity weighted. The Minor Axis Intensity is the narrowest dimension of the ellipse of best fit and is intensity weighted.



The figure below illustrates the difference between intensity weighted and non-intensity weighted Major or Minor Axis and Aspect Ratio. See the Aspect Ratio Intensity Feature for more information.



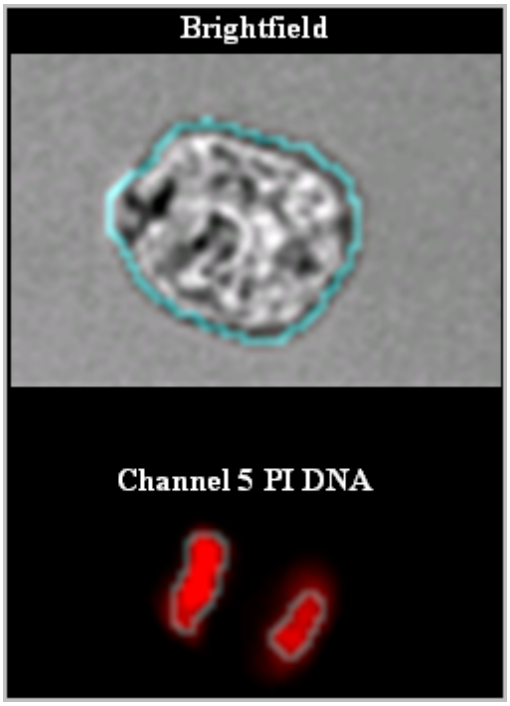
Application Examples:

- Quantify and compare the image fluorescence shape.
- Identify single cells.

Perimeter Feature

The perimeter feature measures the boundary length of the mask in the number of microns.

This example uses a 1-pixel wide mask created to illustrate how a perimeter would appear.



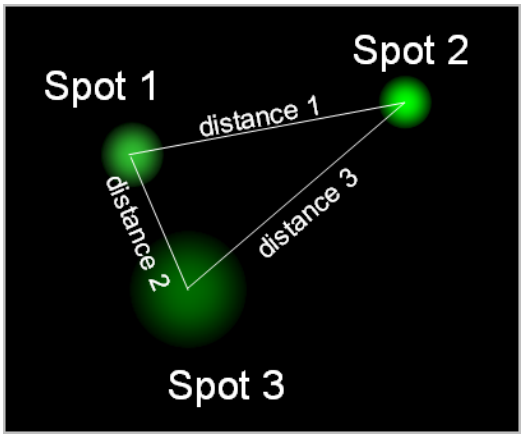
Application Examples:

- Quantify and compare cell circumference.
- Identify cells with highly irregular surfaces from smooth cells.
- Perimeter of the morphology or threshold masks can identify cells with or without dendrites.

Spot Area Min Feature

The Spot Area Min feature provides the area of the smallest spot (connected component) in a spot or peak mask.

This is one of four features that can be used to identify objects with spots that are close together, dim, bright or small when counting spots in an image. To use these features, the spots need to be individually masked, such as using the Spot or Peak Mask. The Spot Area Min, Spot Distance Min, and Spot Intensity Min or Max features measure properties of different spots in an image and are often used with the Spot Count feature (under Texture). For more information see Spot Distance Min FeatureSpot Count FeatureSpot Intensity Min and Spot Intensity Max Features.



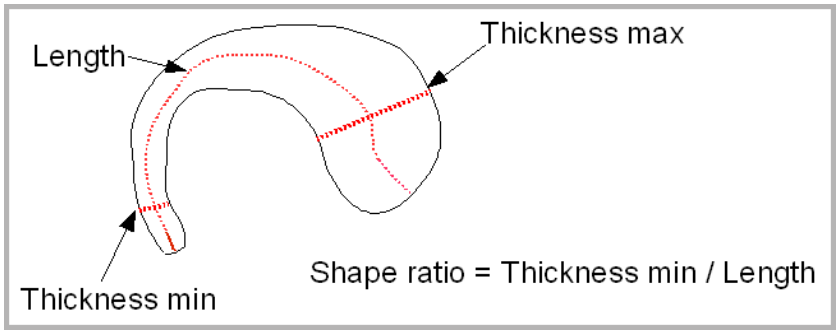
- Spot Area Min is the Area of spot 1
- Spot Distance Min is distance 2 in microns
- Spot Intensity Max is the Raw Mean Pixel of spot 2
- Spot Intensity Min is the Raw Mean Pixel value of spot 3

Application Example:

– In FISH Spot Counting, these features are used to identify objects with ambiguous spots that are located too close together, are too dim to count or are too small in order to remove these objects from the analysis.

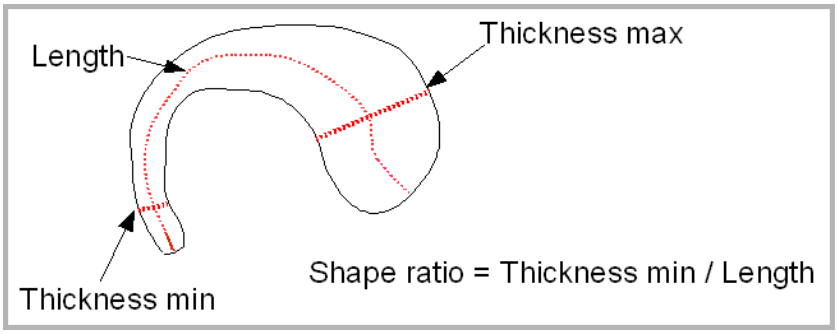
Thickness Max Feature

Thickness Max measures the largest width of an object. This feature is based on an input mask and therefore sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important. See also Table of Base Features- by Category , Length Feature and Shape Ratio Feature for more information.



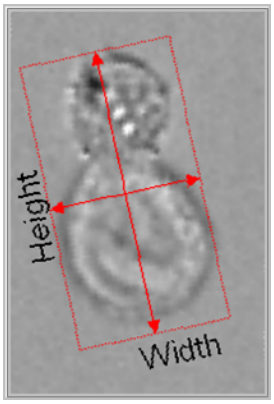
Thickness Min Feature

Thickness Min measures the smallest width of an object. This feature is based on an input mask and therefore sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important. See also Thickness Max Feature, Length Feature and Shape Ratio Feature for more information.



Width Feature

Using the bounding rectangle, Width is the number of microns of the smaller side and Height the longer side. See also Elongatedness Feature.



Application Example:

– These features can be used to separate rectangular shaped objects. For curved objects, measurement is more accurately obtained using the thickness features.

Understanding the Location Features

Location features include Angle, Angle Intensity, Centroid X, Centroid Y, Centroid X Intensity, Centroid Y Intensity, Delta Centroid X, Delta Centroid Y, Delta Centroid XY, Max Contour position, Spot Distance Min, Valley X and Valley Y.

Angle Feature

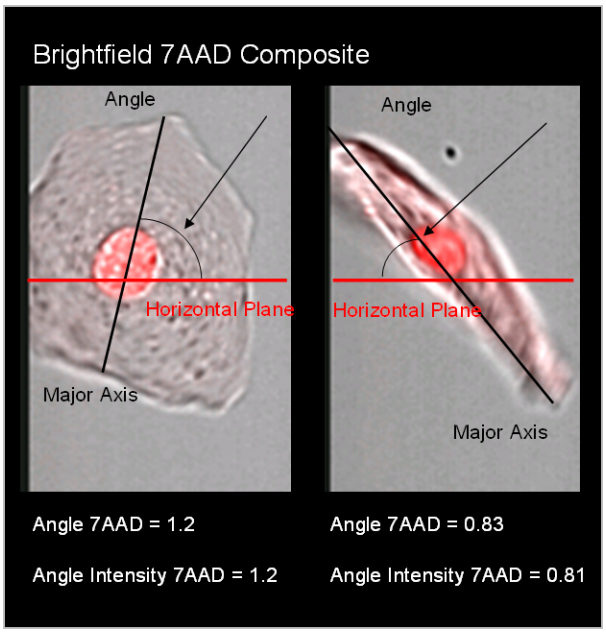
Angle is the angle of the major axis from a horizontal plane in radians.

Application Example:

– Identify the orientation of an image relative to the image frame.

Angle Intensity Feature

Angle Intensity is the angle of the major axis intensity from a horizontal plane in radians.



Application Example:

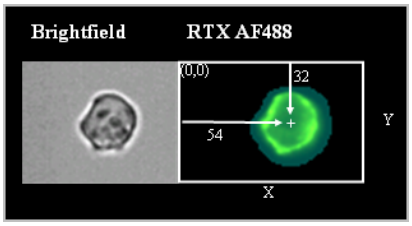
– Identify the orientation of an image relative to the image frame.

Centroid Features

Centroid X and Centroid Y Features

Centroid X is the number of pixels in the horizontal axis from the upper, left corner of the image to the center of the mask. Centroid Y is the number of pixels in the vertical axis from the upper, left corner of the image to the center of the mask.

In this example, the Centroid X=54 and the Centroid Y=32.

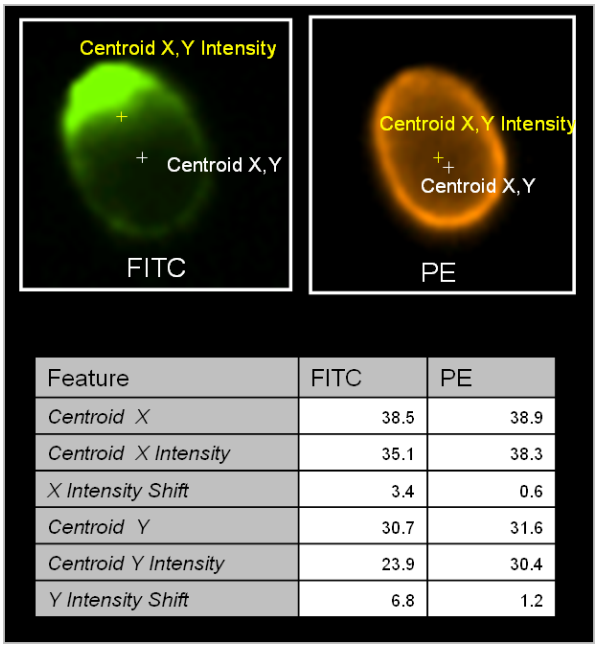


Application Examples:

- Identify the center of the mask.
- Calculate the Delta Centroid or the distance between two fluorescent markers.
- Used by IDEAS to calculate the Delta Centroid X, Y, or XY.

Centroid X Intensity and Centroid Y Intensity Features

Centroid X Intensity is the intensity weighted X centroid and is shifted from the center of the mask toward the center of fluorescence. The Centroid Y Intensity is the intensity weighted Y centroid. X and Y pixel coordinates are calculated from an origin in the upper left corner.



Feature	FITC	PE
Centroid X	38.5	38.9
Centroid X Intensity	35.1	38.3
X Intensity Shift	3.4	0.6
Centroid Y	30.7	31.6
Centroid Y Intensity	23.9	30.4
Y Intensity Shift	6.8	1.2

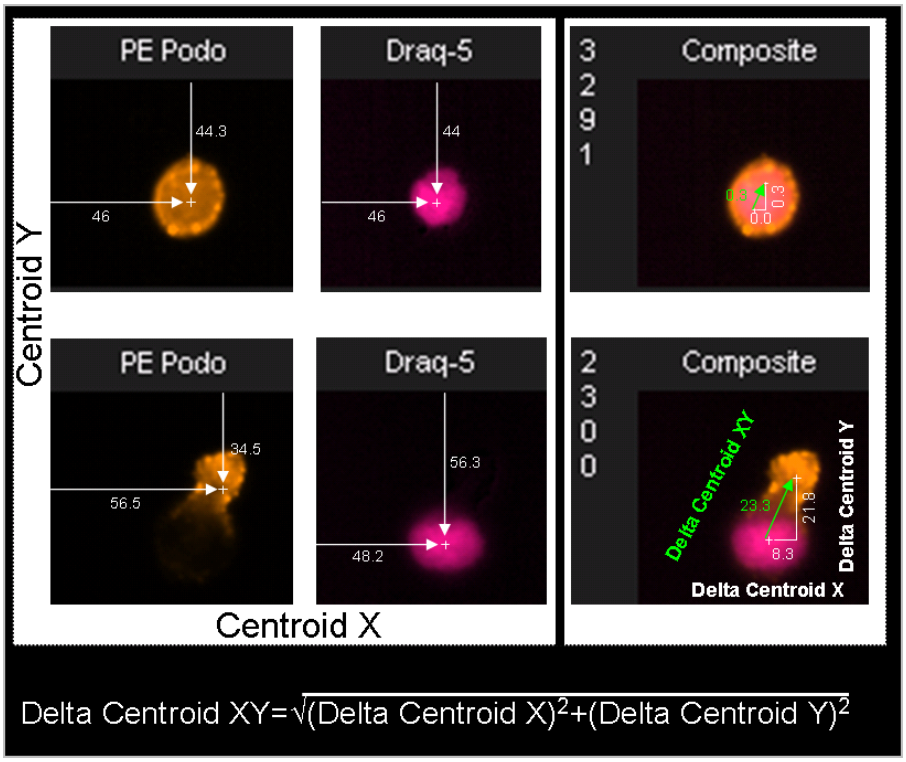
Application Examples:

- Identify the center of peak fluorescence.
- Calculate the distance between two fluorescent markers.
- Used by IDEAS to calculate the intensity weighted Delta Centroid X, Y or XY.

Delta Centroid X and Delta Centroid Y Features

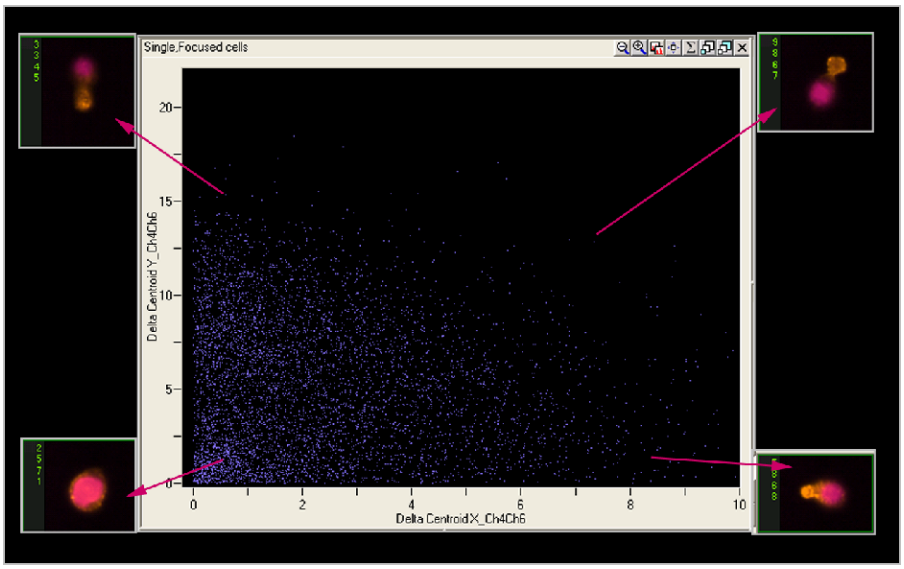
Both the Delta Centroid X and Y features measure the distance between the Centroids X or Centroids Y, respectively, of two images using the user-provided masks for each image. Either one or both the centroids of the images may be intensity-weighted. X and Y pixel coordinates are calculated from an origin in the upper left corner to obtain the centroid positions and the distance between the centroids is converted to microns.

An example is shown below.



The graph below illustrates using the Delta Centroid X versus Delta Centroid Y to identify cells with a variation of location of a protein with respect to the nucleus.

Cells with no spatial shift of signal between the nuclear stain(Ch6) and protein of interest(Ch4) have a low Delta Centroid X and Y and are found in the lower left corner. Cells with a large shift between the images in both the X and Y direction are found in the upper, right section and those with a large shift in X but not Y are found in the lower, right. Similarly a cell with a large shift in the Y direction and not X are found in the upper, left. See Delta Centroid XY Feature to measure the X and Y shift together.

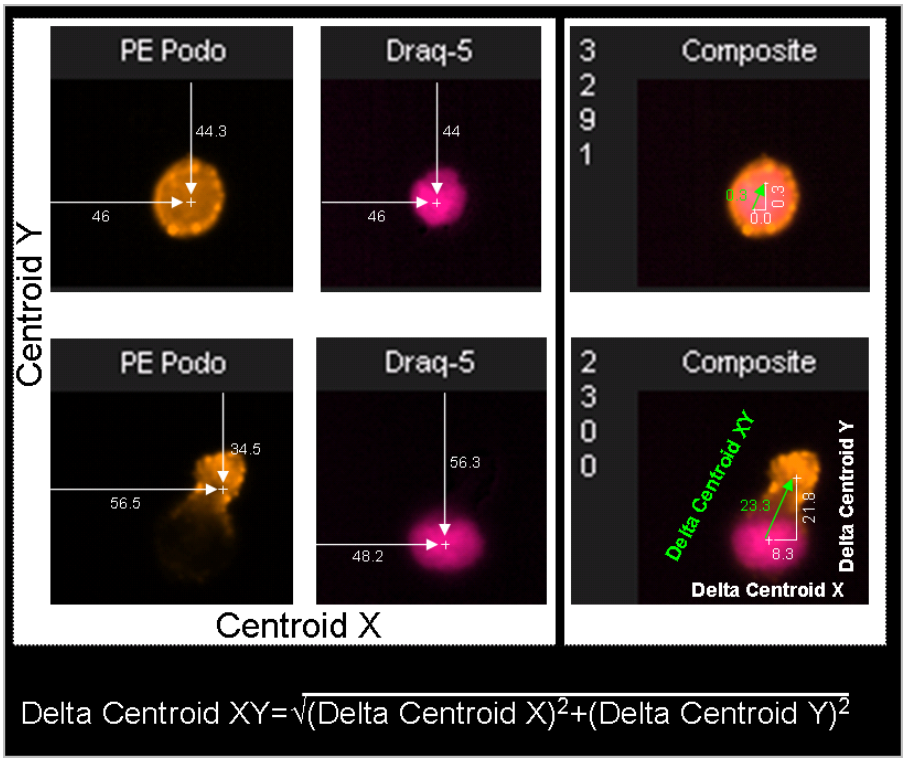


Application Example:

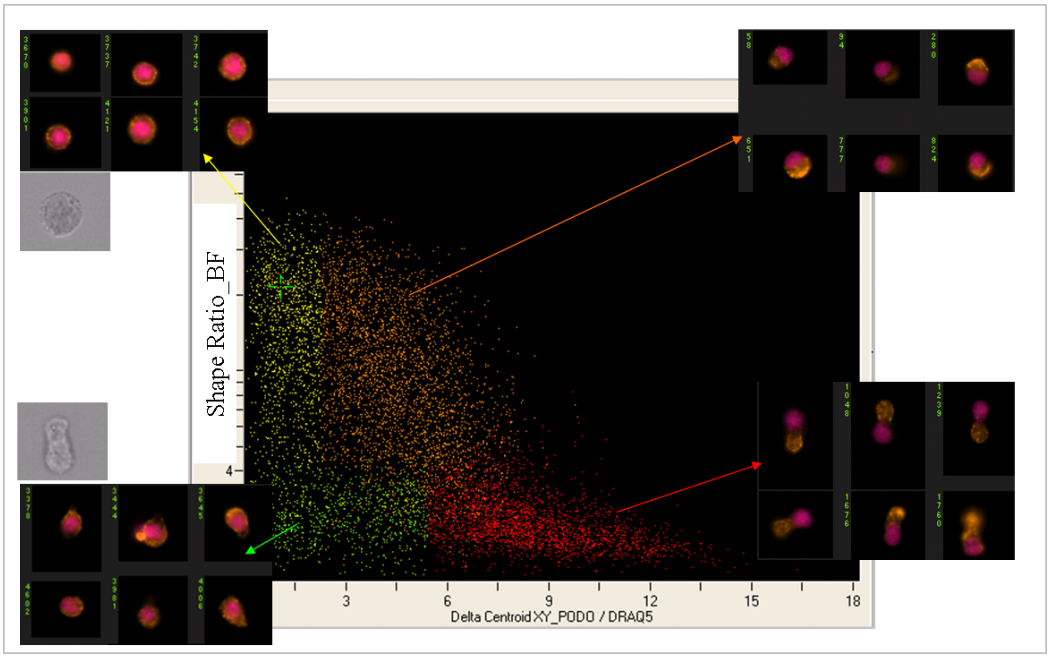
- Used to identify capped versus not capped cells.
- Used to measure shifts in X or Y direction between two images.

Delta Centroid XY Feature

The Delta Centroid XY feature measures the distance between the Centroid feature of two images using the user-provided masks for each image. Either one or both the centroids of the images may be intensity-weighted. X and Y pixel coordinates are calculated from an origin in the upper left corner to obtain the centroid positions and the distance between the centroids is converted to microns. In the example, below an image pair is shown stained with the nuclear dye Draq 5 and a PE labeled antibody that is differentially expressed two cells, either uniformly or in the pseudopod. The two cells are identified by their different Delta Centroid XY values.



Below is an example of using the Delta Centroid XY. A bivariate graph of a shape ratio versus Delta Centroid XY can identify cells with caps as shown here:



Application Examples:

– Quantify the spatial relationship between two fluorescent probes.

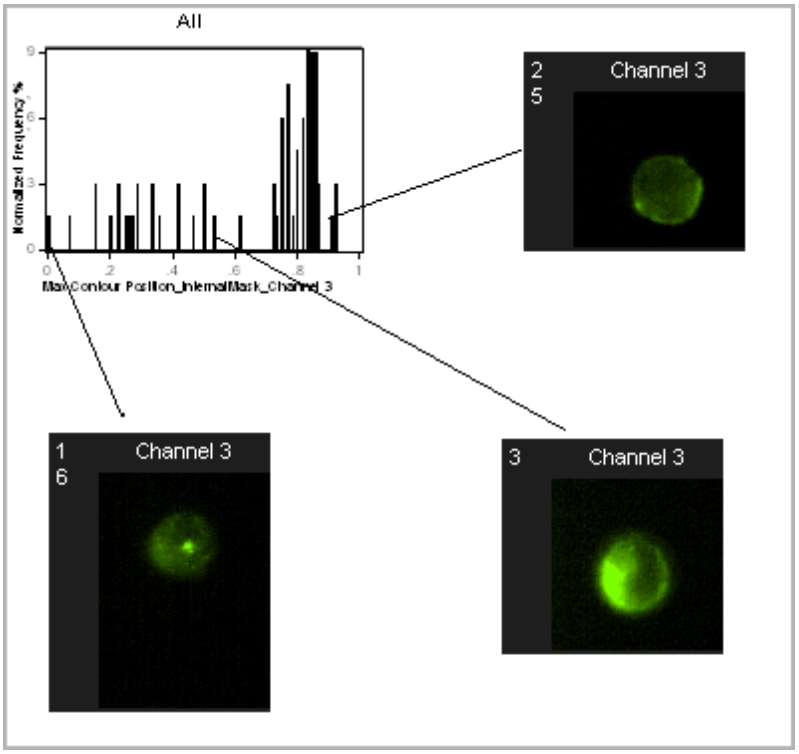
- Identify false apoptotic positive cells in the TUNEL and Annexin V assays.
- Quantify shape change.
- Quantify capping of cell surface antigens.

Raw Centroid X and Raw Centroid Y Features

The centroid X and Y of the original position of the image during acquisition before it was centered IDEAS. Data analyzed in IDEAS versions 4.0 or later cut and center objects that were collected as one image in INSPIRE.

Max Contour Position Feature

The Max Contour Position is defined as the location of the contour in the cell that has the highest intensity concentration. It is invariant to object size and can accommodate localized intensity concentrations. The actual location in the object is mapped to a number between 0 and 1, with 0 being the object center and 1 being the object perimeter, which allows one to compare the results across cells of different sizes. An example is shown below.



Application Example:

– Used in conjunction with the Internalization feature to determine the distribution of intensity within a cell.

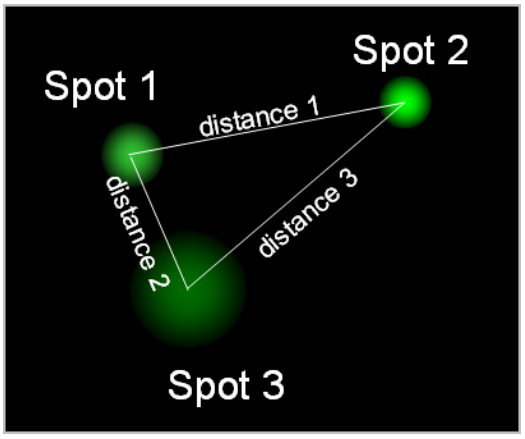
Shift X and Shift Y Features

The Shift X or Shift Y feature is the location of the highest cross-correlation of a pair of images. When two identical images are aligned perfectly the cross-correlation is at it's maximum. The shift X or Shift Y is the shift required to get the highest cross-correlation value for the 2 images. This feature is used mainly for troubleshooting cross-camera alignment.

Spot Distance Min Feature

The Spot Distance Min feature provides the shortest distance in microns between two spots (connected components) in a spot or peak mask.

This is one of four features that can be used to identify objects with spots that are close together, dim, bright or small when counting spots in an image. To use these features, the spots need to be individually masked, such as using the Spot or Peak Mask. The Spot Area, Distance, and Spopt Intensity Min or Max features measure properties of different spots in an image and are often used with the Spot Count feature (under Texture). For more information see Spot Area Min Feature; Spot Count Feature; Spot Intensity Min and Spot Intensity Max Features.



- Spot Area Min is the Area of spot 1
- Spot Distance Min is distance 2 in microns
- Spot Intensity Max is the Raw Mean Pixel of spot 2
- Spot Intensity Min is the Raw Mean Pixel value of spot 3

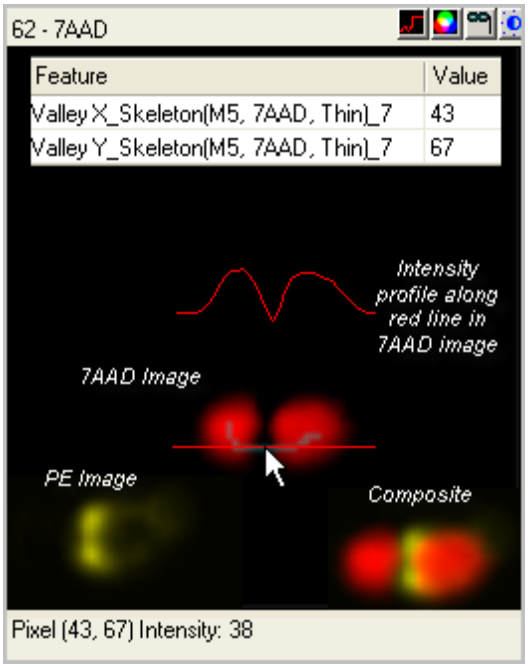
Application Example:

– In FISH Spot Counting, these features are used to identify ambiguous spots that are located too close together, too dim, to bright or too small to count and can be eliminated from the analysis.

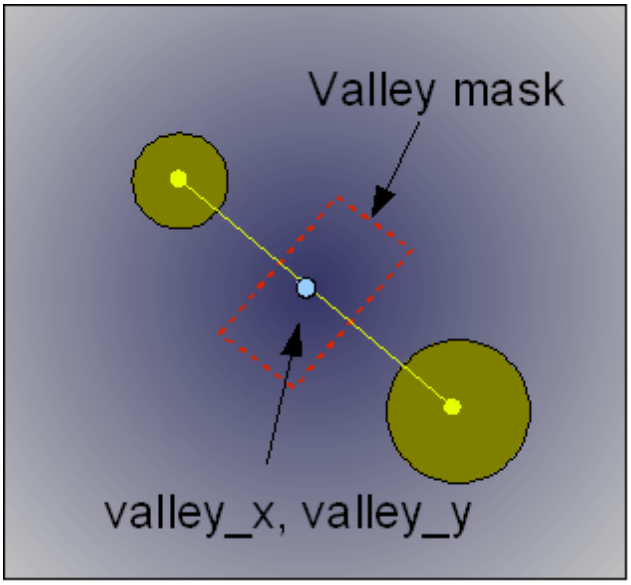
Valley X and Valley Y Features

The Valley X and Y are the exact X,Y coordinates of the minimum intensity within the skeletal lines of the input mask. The objects condensed shape, typically 1-pixel wide skeletal line is determined from the starting mask. This is also the origin of the Valley mask. See Valley Mask and Skeleton Mask.

In the figure below, the Valley X and Valley Y position of the 7AAD image is shown. In this example a protein of interest in the PE image localizes to the synapse between two cells.



These features define the origin of the Valley mask.



Application Example:

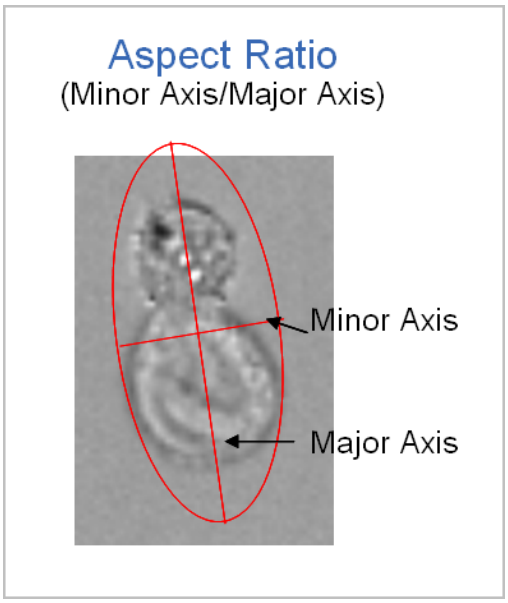
– Measure the exact center of where a synapse between two cells is located.

Understanding the Shape Features

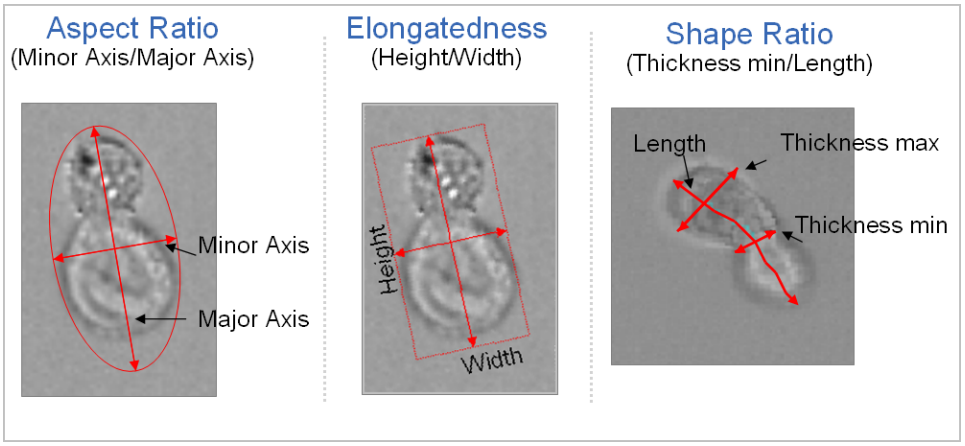
Shape features define the mask shape and have units that vary with the feature. They include the Aspect Ratio, Aspect Ratio Intensity, Compactness, Elongatedness, Lobe Count, and Symmetry 2,3,4.

Aspect Ratio Feature

Aspect Ratio is the Minor Axis divided by the Major Axis and describes how round or oblong an object is. See also: Major Axis and Minor Axis Features.



See also Elongatedness Feature and Shape Ratio Feature for other shape ratios.



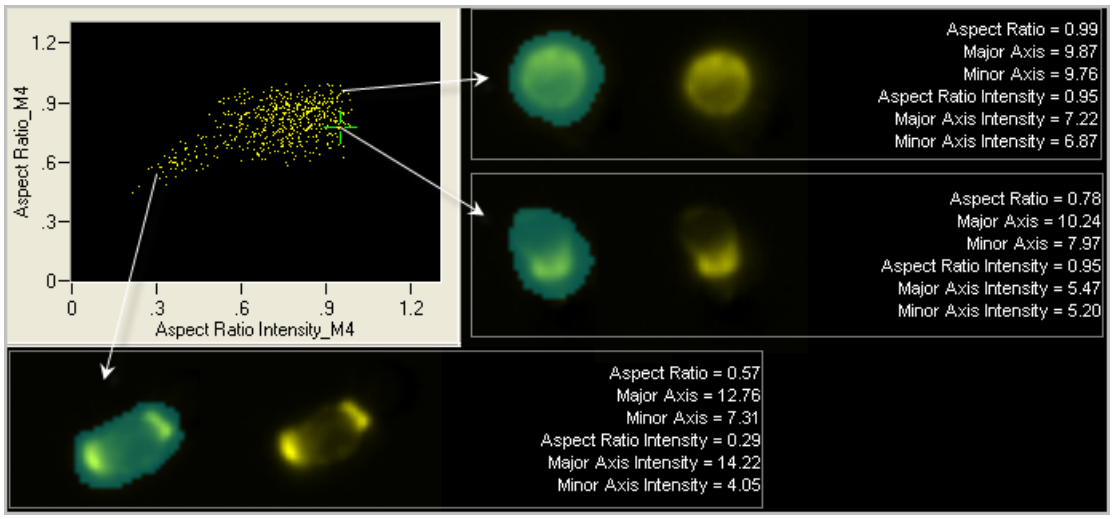
Application Examples:

- Quantify the roundness of the mask.
- Identify single cells vs. doublets.
- Cell classification based on shape change.
- Identify recently divided cells in mitosis.

Aspect Ratio Intensity Feature

Aspect Ratio Intensity is the Minor Axis Intensity divided by the Major Axis Intensity. See also: Major Axis Intensity and Minor Axis Intensity Features.

The figure below illustrates the difference between Aspect Ratio Intensity and Aspect Ratio. See also: Aspect Ratio Feature.

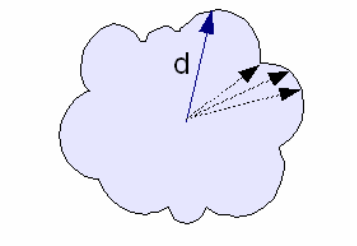


Application Examples:

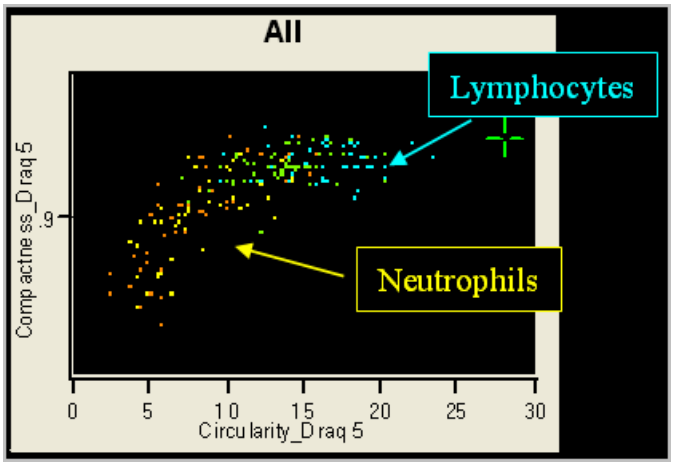
- Quantify the roundness of the fluorescent image.
- Better resolution for identifying single cells vs. doublets in experiments using a DNA dye.
- Cell classification based on fluorescent morphology.

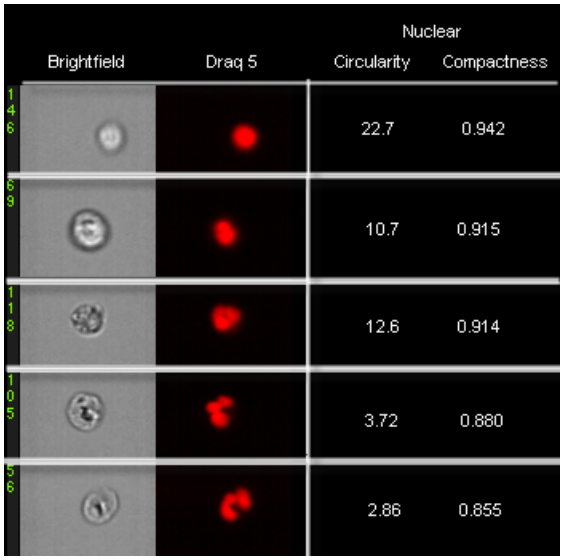
Circularity Feature

This feature measures the degree of the mask's deviation from a circle. Its measurement is based on the average distance of the object boundary from its center divided by the variation of this distance. Thus, the closer the object to a circle, the smaller the variation and therefore the feature value will be high. Vice versa, the more the shape deviates from a circle, the higher the variation and therefore the Circularity value will be low. See also .



Below is an example using Circularity and Compactness to characterize the shape of peripheral blood mononuclear cells stained with the DNA dye Draq 5.





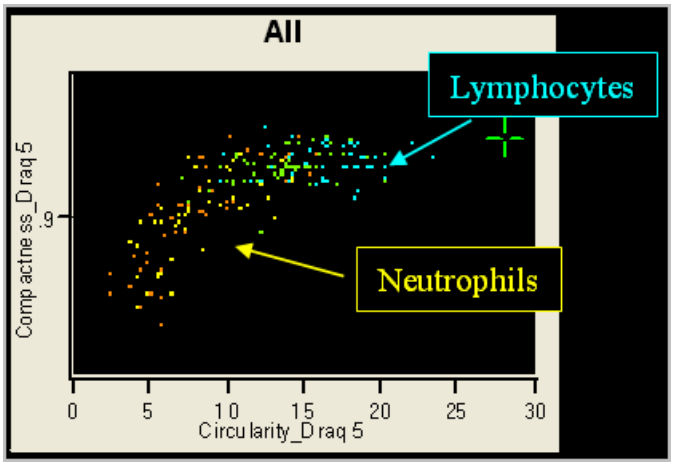
Application Examples:

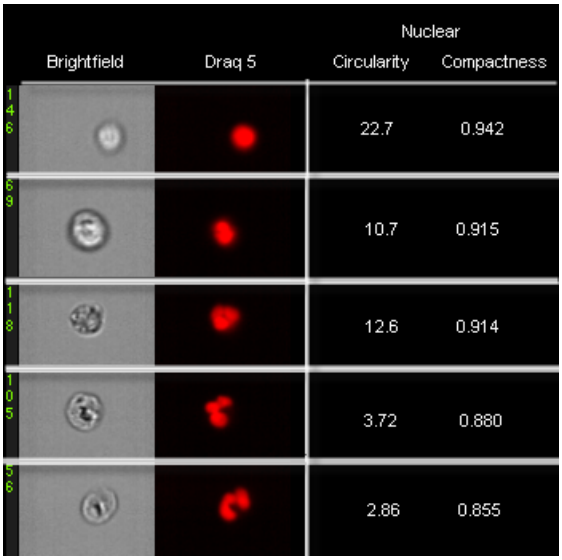
- Distinguish singlets and doublets.
- Separate circular and non circular shapes.
- Used in the Shape Change Wizard

Compactness Feature

Compactness measures the degree of how well the object is packed together. This feature is similar to the Circularity feature but unlike Circularity, this feature includes all of the pixels within the mask and is intensity weighted. The higher the value, the more condensed the object.

Below is an example using Circularity and Compactness to characterize the shape of peripheral blood mononuclear cells stained with the DNA dye Draq 5.



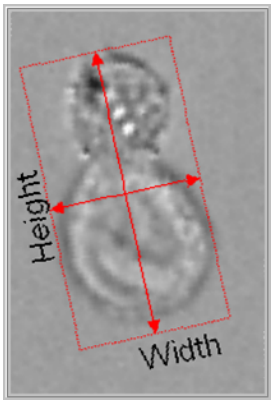


Application Example:

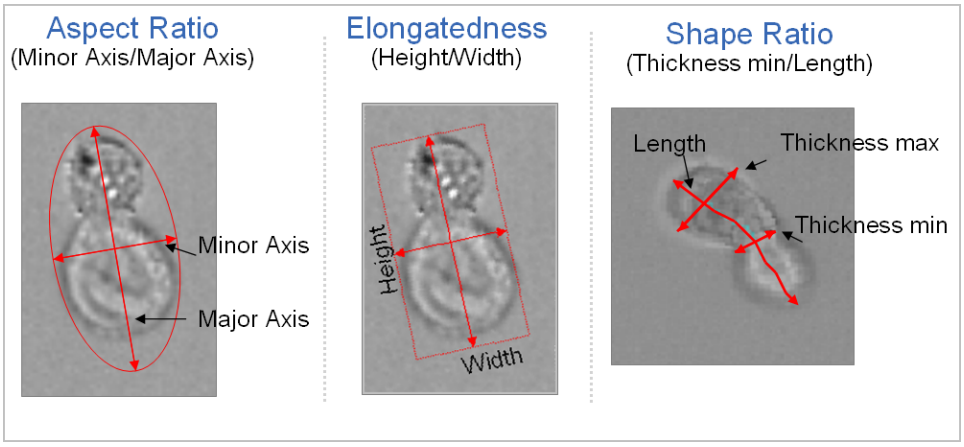
– Differentiate between rounded objects with smooth boundary to less regular objects.

Elongatedness Feature

Elongatedness is the ratio of the Height over Width of the object's bounding box. See also Width Feature.



See also Aspect Ratio Feature and Shape Ratio Feature for other shape ratios.



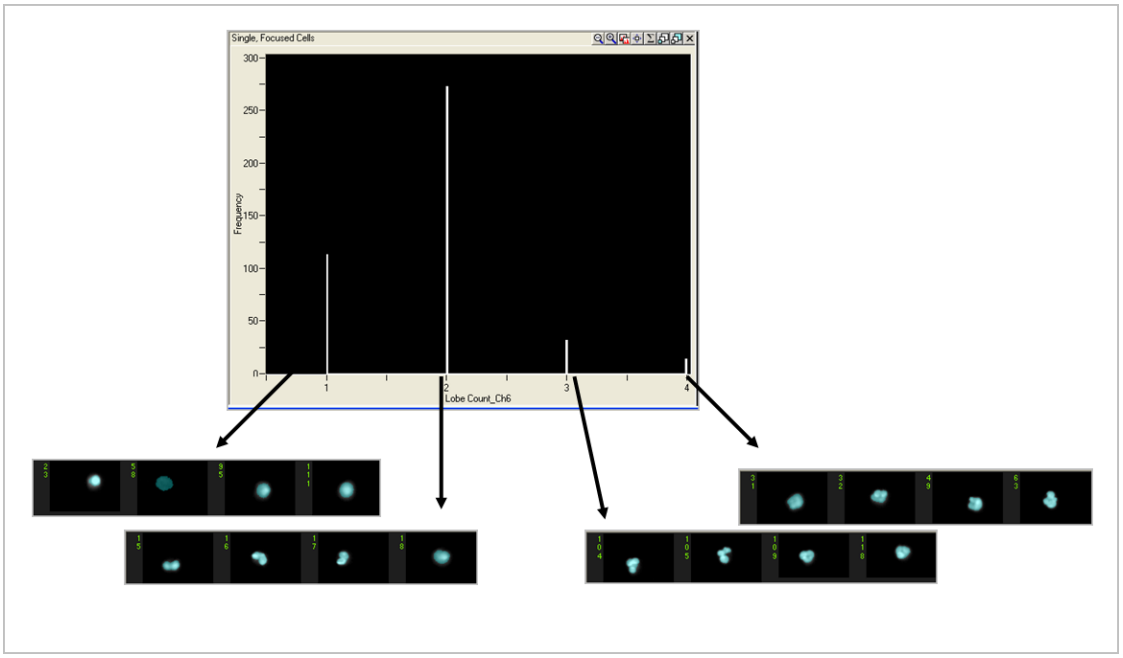
Application Examples:

- Measure object shape properties to differentiate between long and narrow versus short and thick objects.
- Quantify the roundness of the morphology mask.
- Identify single cells and doublets.
- Cell classification based on shape change.
- Identify recently divided cells in mitosis.

Lobe Count Feature

The Lobe Count feature counts the number of lobes in a cell. It is determined based on the maxima of the weighted Symmetry features. The feature reports the values 1, 2,3 or 4. If an object does not have a high value for Symmetry 2, Symmetry 3, or Symmetry 4 it is reported as 1 for no lobes. An example is shown below. See also Symmetry 2, 3, 4 Features.

	Symmetry		
Lobe Count	2	3	4
1	Low	Low	Low
2	High	Low	Low
3	Low	High	Low
4	Low	Low	High



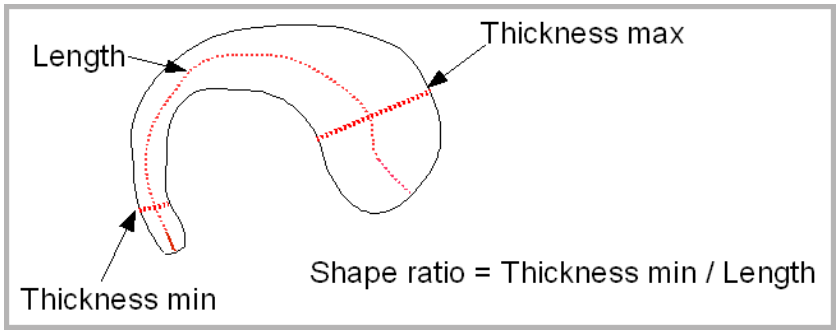
Application Example:

– Used in cell classification studies. Also used to differentiate small round cells from small square cells of similar area.

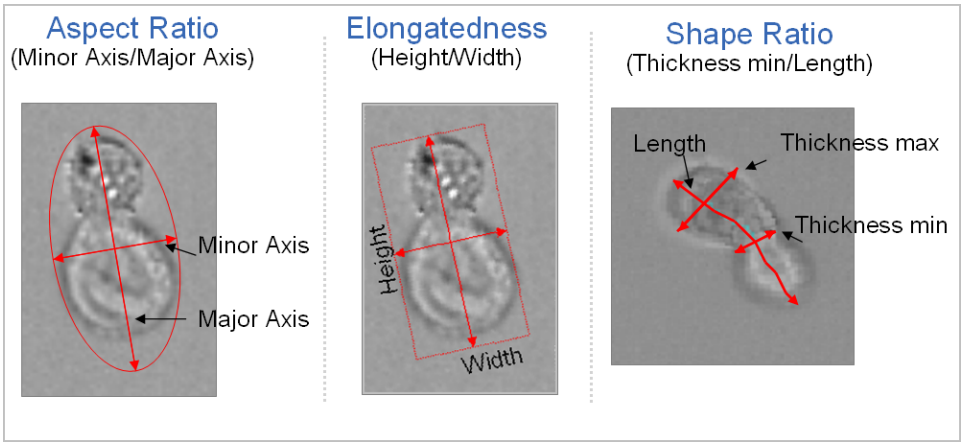
Shape Ratio Feature

The Shape Ratio is Thickness Min divided by Length.

The Shape Ratio feature is based on an input mask and is sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important.



See also Aspect Ratio Feature and Elongatedness Feature for other shape ratios.

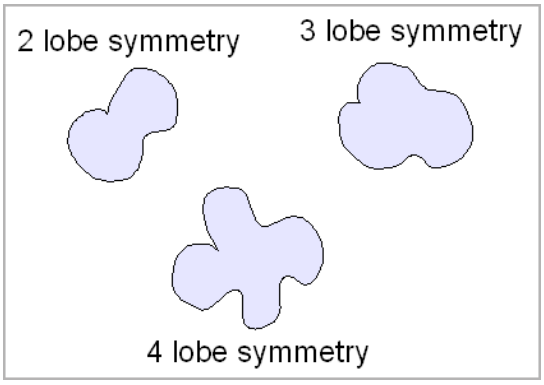


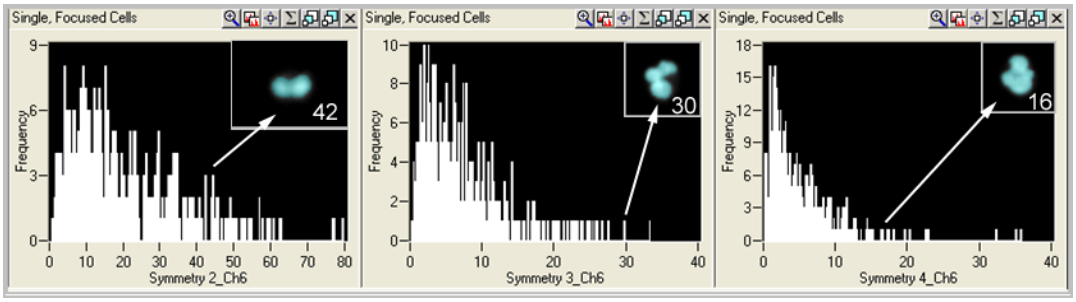
Application Example:

– Measure object's elongatedness to provide shape classification.

Symmetry 2, 3, 4 Features

The Symmetry 2 feature measures the tendency of the object to have a single axis of elongation and therefore 2 lobes. The Symmetry 3 feature measures the tendency of the object to have a three-fold axis of symmetry and likewise, Symmetry 4 a four-fold axis. The absolute value of these features are dependent on the number of lobes. For example an image that has high 4 lobe symmetry will also have high 2 lobe symmetry. See the Lobe Count Feature for more information.





Application Example:

– Classify different white blood cells based on the morphology of the nuclear image.

Understanding the Texture Features

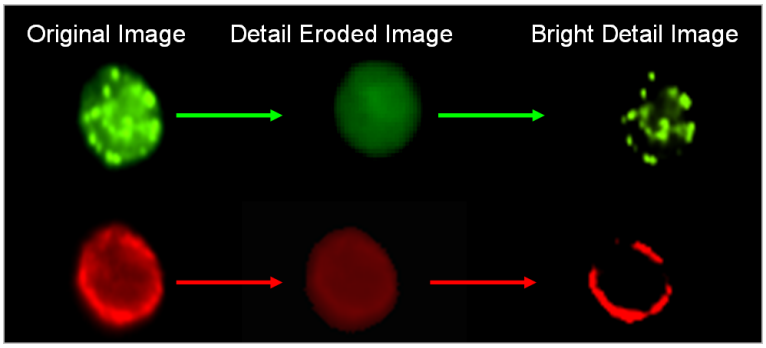
The Texture features determine local intensity variations in images and include Bright Detail Intensity R3 and Bright Detail Intensity R7, Contrast, Gradient Max, Gradient RMS, H Texture (H-Contrast, H-Correlation, H-Energy, H-Entropy, H-Homogeneity, and H-Variance), Modulation, Spot Count, and Std Dev.

Contrast, Gradient Max and Gradient RMS are generally used to determine best focus.

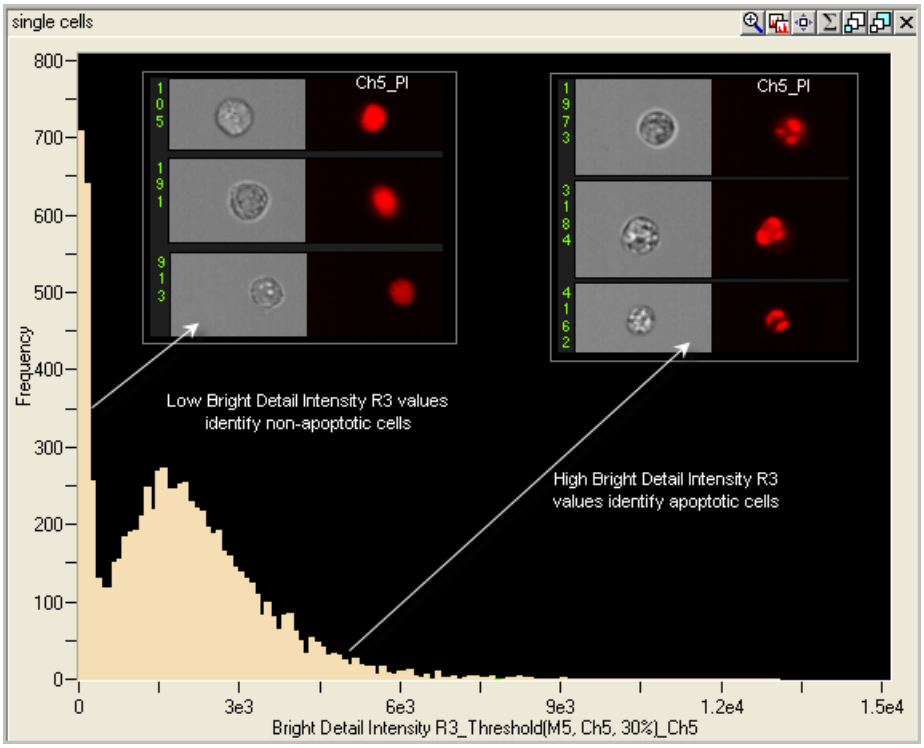
Bright Detail Intensity R3 and Bright detail Intensity R7 Features

The Bright Detail Intensity R3 and Bright Detail Intensity R7 features compute the intensity of localized bright spots within the masked area in the image. Bright Detail Intensity R3 computes the intensity of bright spots that are 3 pixels in radius or less, while Bright Detail Intensity R7 computes the intensity of bright spots in the image that are 7 pixels in radius or less. In each case, the local background around the spots is removed before the intensity computation.

The figure below shows the process of obtaining the localized bright spots in the image.



The graph below illustrates the use of the Bright Detail Intensity R3 feature on a nuclear image to separate apoptotic cells from non-apoptotic cells.

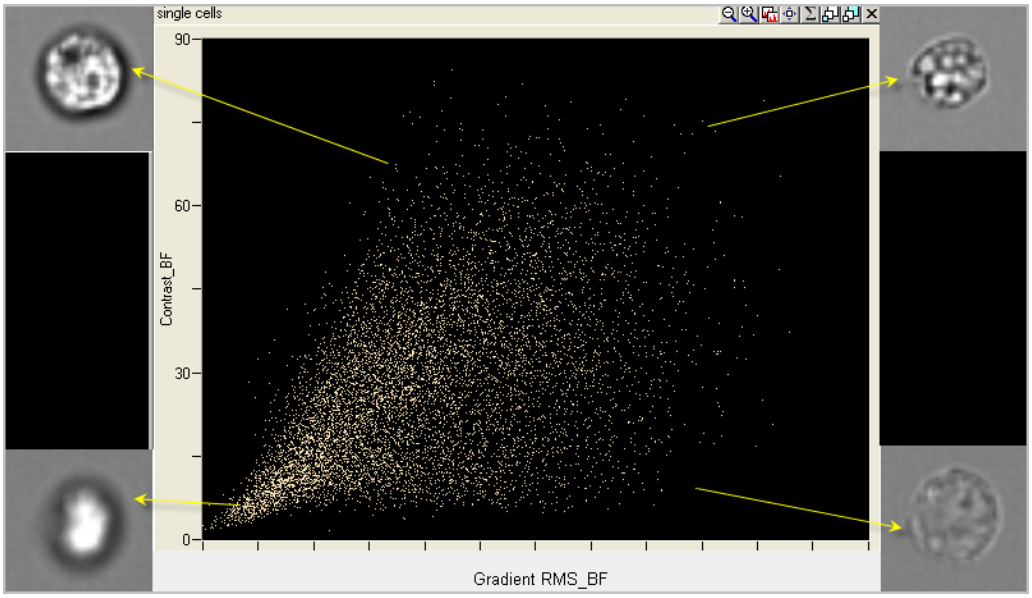


Application Example:

- Identify cells that have bright specks such as Apoptotic cells.
- Used in the Cell Cycle - Mitosis Wizard.

Contrast Feature

The Contrast feature measures the sharpness quality of an image by detecting large changes of pixel values in the image and is useful for the selection of focused objects or apoptotic brightfield images. For every pixel, the slopes of the pixel intensities are computed using the 3x3 block around the pixel. This is similar to the Gradient RMS calculation with different weighted assignments to the pixel arrays with no background subtraction. Example images are shown in the figure below.



Application Examples:

- Find apoptotic images with high contrast in brightfield imagery.
- Determine overall focus quality of images.
- Use with Gradient RMS to determine focus quality.
- Characterize texture.

See also: Gradient Max Feature and Gradient RMS Feature.

Ensquared Energy Feature

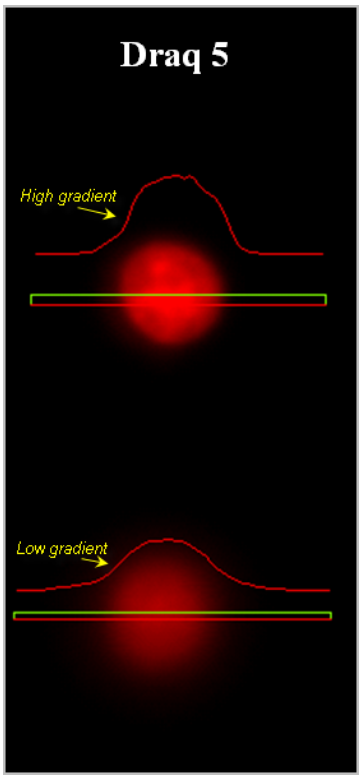
The Ensquared Energy feature is a measure of image quality. Computes the intensity of the square block around the brightest pixel using the diameter input as the side for the square divided by the intensity of the total intensity. The closer this ratio is to 1.0 the better focused the image. This feature is mainly used for single, uniform particles such as beads.

The figure below shows the image quality test using the Ensquared Energy feature.

Gradient Max Feature

The Gradient Max feature measures the sharpness quality of an image by detecting largest change of pixel values in the image and is useful for the selection of focused objects.

This figure shows the change in intensity across the red line. The top image has a larger slope change than the lower image.



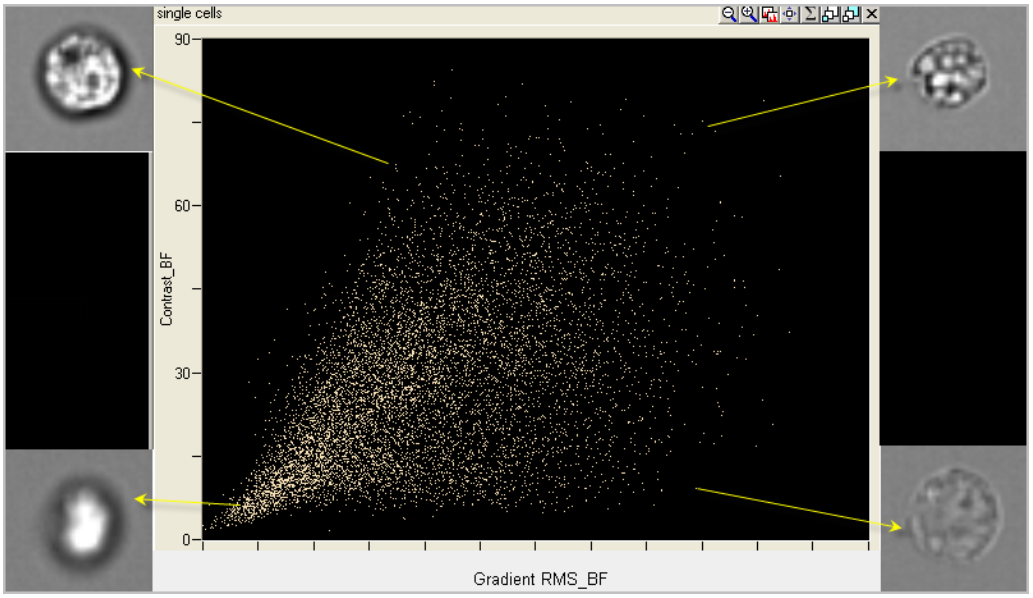
Application Example:

- Determine peak focus quality of images.
- Also used to characterize texture. However, the Gradient RMS and Contrast feature are more robust for these applications.

See also: Gradient RMS Feature and Contrast Feature.

Gradient RMS Feature

The Gradient RMS feature measures the sharpness quality of an image by detecting large changes of pixel values in the image and is useful for the selection of focused objects. The Gradient RMS feature is computed using the average gradient of a pixel normalized for variations in intensity levels. This is similar to the Contrast calculation with different weighted assignments to the pixel arrays and with background subtracted. Example images are shown in the figure below.



Application Examples:

- Determine overall focus quality of images.
- Used with Contrast to determine focus quality.
- Characterize texture.

See also: Gradient Max Feature and Contrast Feature.

H Texture Features

H Texture features include the following: H Energy Mean and Std, H Entropy Mean and Std, H Contrast Mean and Std, H Homogeneity Mean and Std, H Correlation Mean and Std, H Variance Mean and Std Features.

R.M. Haralick (H) defined a set of texture features that characterize the spatial relationships amongst the pixel values in an image[1]. IDEAS uses a common normalization method so that images with different intensities can be directly compared. For each H texture feature, the mean reflects the average value and the standard deviation (Std) reflects the presence of texture orientation.

The user defines the texture grain by assigning a granularity value. For very fine textures, this value is small (1-3 pixels), while for very coarse textures, it is large (>10). In the IDEAS default template, the granularity value is 5.

While these features have value for distinguishing cellular texture when used individually, images often contain a mixture of different textures at different grains. Therefore, these features are most powerful when combined.

Application Example:

- Quantify texture in cells.

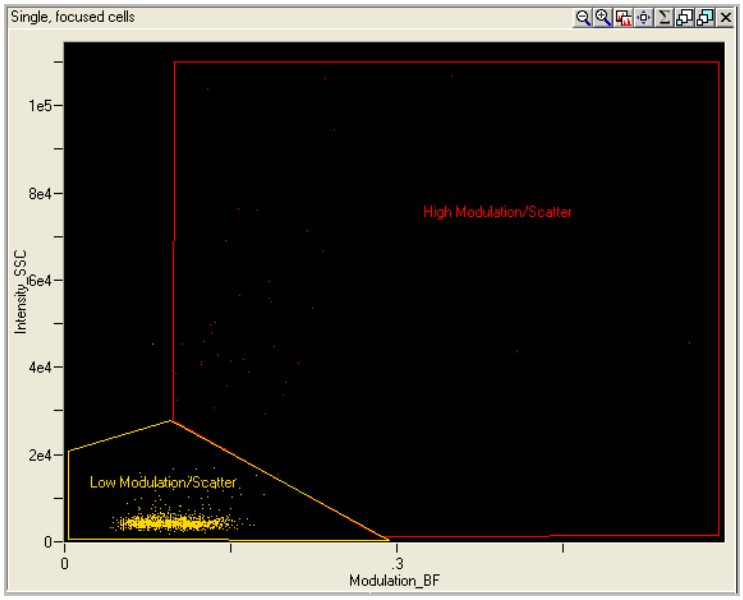
[1] Haralick, R.M., K. Shanmugan, and I. Dinstein, "Textural Features for Image Classification", *IEEE Transactions on Systems, Man, and Cybernetics*, Vol. SMC-3, 1973, pp. 610-621.

Modulation Feature

The Modulation feature measures the intensity range of an image, normalized between 0 and 1.

The formula is: Modulation = Max Pixel - Min Pixel / Max Pixel + Min Pixel

The following example illustrates Modulation on brightfield images and Intensity of scatter in channel 1.



High Modulation

Low Modulation

Low Modulation

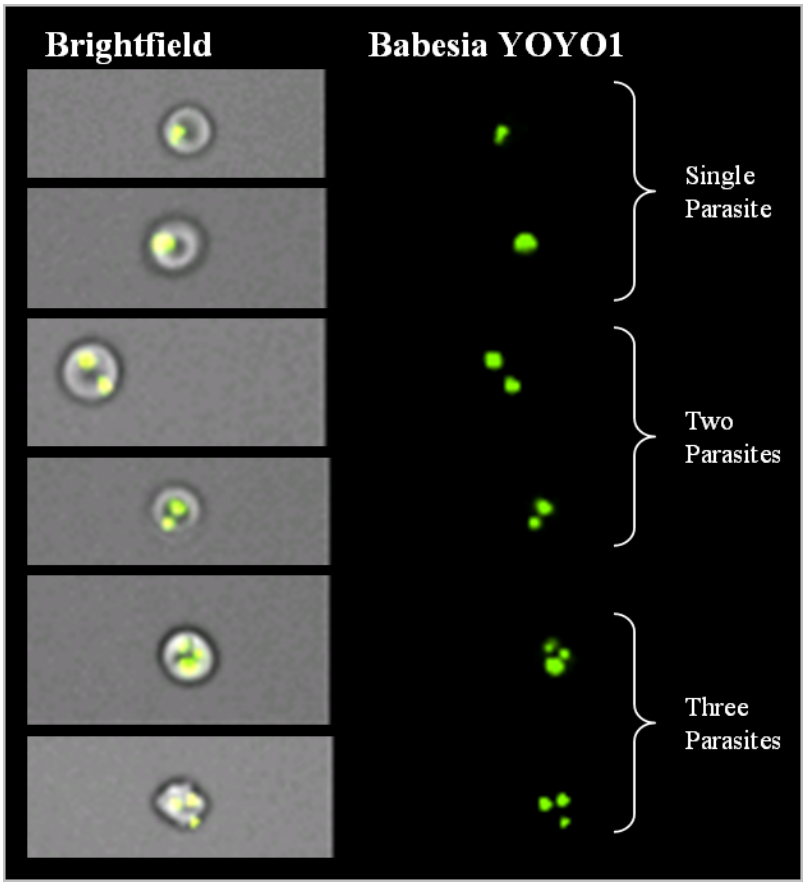
Application Example:

- Quantify image quality and characterize contrast and texture in cells.

Spot Count Feature

The Spot Count feature provides the number of connected components in an image. The connected component algorithm examines the connectivity of each pixel based on whether this pixel is connected to a particular spot or the background. In order to count the number of connected components the mask input is very important. See Spot Mask, Peak Mask, and Range Mask for information on masking spots. See also Spot Area Min Feature, Spot Distance Min Feature, andSpot Intensity Min and Spot Intensity Max Features for more information.

The following figure illustrates the application of Spot Counting to quantify parasitic infection of Babesia in erythrocytes by staining nuclei with YOYO (green).



Application Examples:

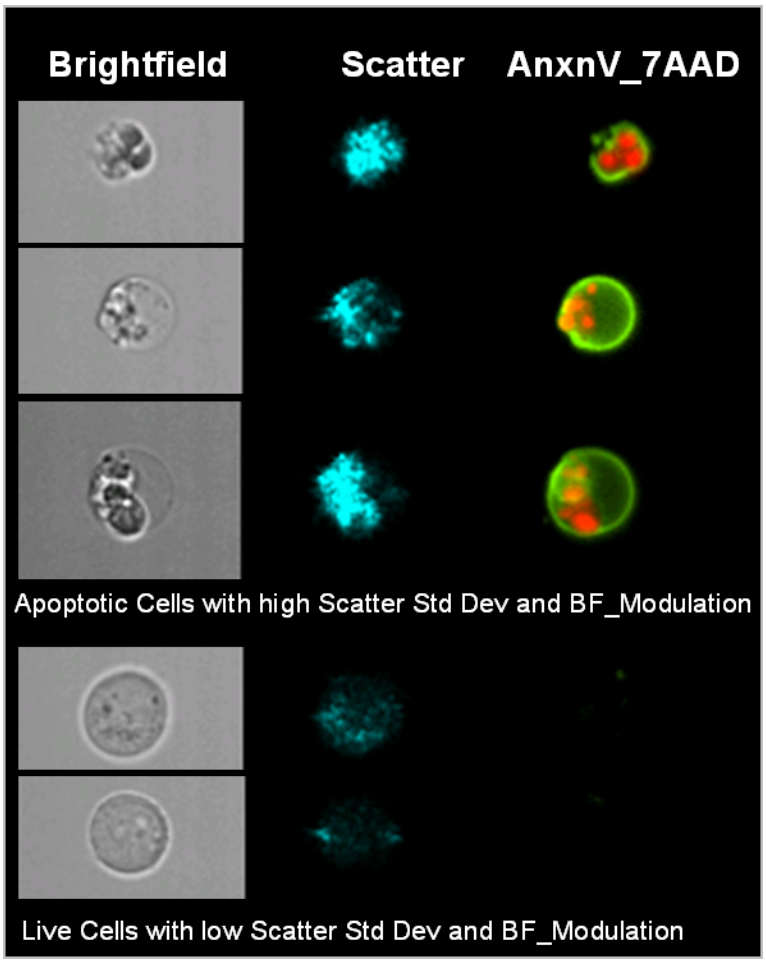
- Counting parasites.
- Counting phagocytosed particles.
- FISH spot counting.
- Counting punctate spots in images.
- Used in the Spot Wizard.

Std Dev Feature

The Std Dev feature describes the overall distribution of pixel intensities.

The Std Dev is the standard deviation of the pixel intensity values in the mask. The Std Dev value provides an indication of the texture or complexity of an object.

The following illustrates that apoptotic cells (AnxnV positive) exhibit higher Std Dev values in the darkfield channel (scatter) and higher brightfield Modulation values than non-apoptotic cells (AnxnV negative).


Brightfield
Scatter
AnxnV_7AAD
Apoptotic Cells with high Scatter Std Dev and BF_Modulation
Live Cells with low Scatter Std Dev and BF_Modulation

Application Example:

- Quantify intensity variation within a mask.
- Distinguish apoptotic and necrotic cells.

Understanding the Signal Strength Features

Signal Strength features include the following:

- Bkgd Mean and Bkgd StdDev features describe the background of the image.
- Intensity and Raw Intensity features quantify the intensities in the region of interest.
- Raw Max, Raw Min, Raw Mean and Raw Median Pixel report single pixel values in an image.
- Max, Min, Mean and Median Pixel report background subtracted single pixel values in an image.
- Saturation Count and Saturation Percent quantify the saturated pixels.
- Spot Intensity Min is used when counting spots.

Note that when the name includes ‘Raw’, this means that there is no background subtraction.

Bkgd Mean Feature

The Bkgd Mean feature estimates the average camera background level in an image by taking the mean of the background pixels.

Application Examples:

- Obtain estimate of the mean camera background level.
- Compute background-subtracted pixel values in other feature computations.

Bkgd StdDev Feature

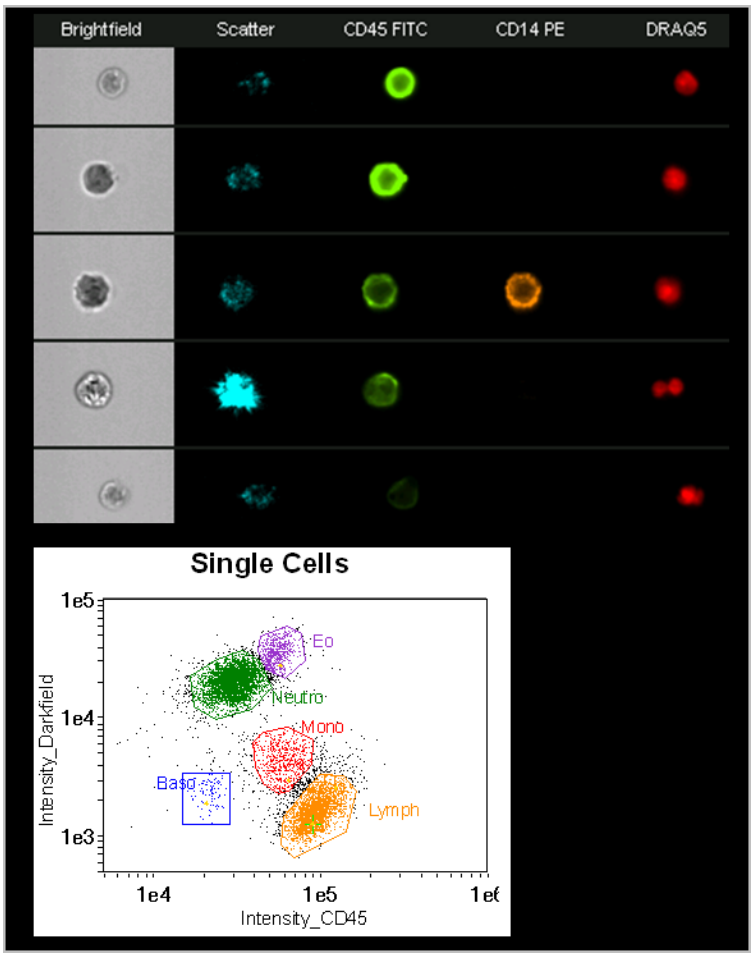
The Bkgd Std Dev feature estimates the standard deviation of the camera background level in an image computed using the background pixels.

Application Example:

– Obtain estimate of the camera background noise.

Intensity Feature

The Intensity feature is the sum of the background subtracted pixel values within the masked area of the image.



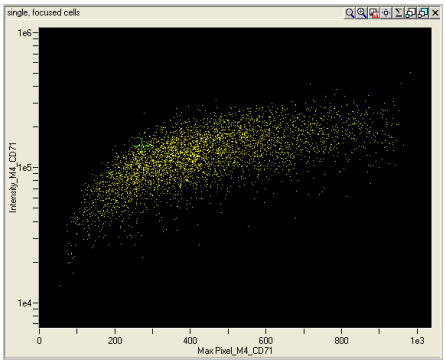
Application Examples:

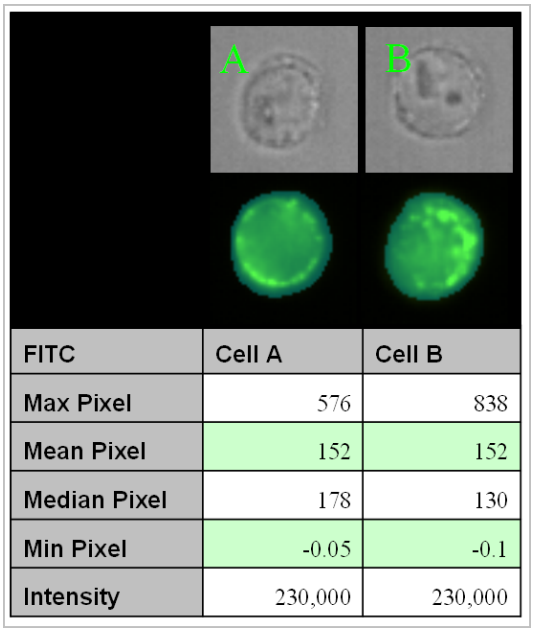
- Quantify relative levels of fluorescence between cells and within different regions of the same cell.
- Immunophenotyping.
- Cell cycle analysis.
- Protein expression.
- Protein activation.

Max Pixel Feature

The Max Pixel feature is the largest value of the background-subtracted pixels contained in the input mask. An example plot is shown below that demonstrates the advantage of using this feature over the Intensity feature for identifying true positive events. For a concentrated signal, Max Pixel is more sensitive than Intensity as shown in the figure below.

The relationship of Max, Mean, Median, and Min Pixel is shown in the figure below:





FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

Application Examples:

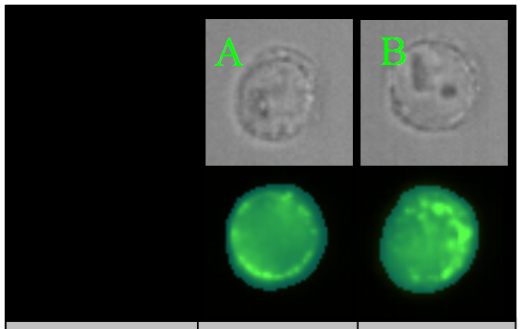
– Used to estimate the true peak fluorescence activity. Is preferred over the Raw Max Pixel for this application.

– Max Pixel to Mean Pixel ratio identifies bright punctate staining vs. uniform staining.

Mean Pixel Feature

The Mean Pixel feature is the mean of the background-subtracted pixels contained in the input mask. This is computed as Intensity/number of pixels.

The relationship of Max, Mean, Median and Min Pixel is shown in the figure below:



FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

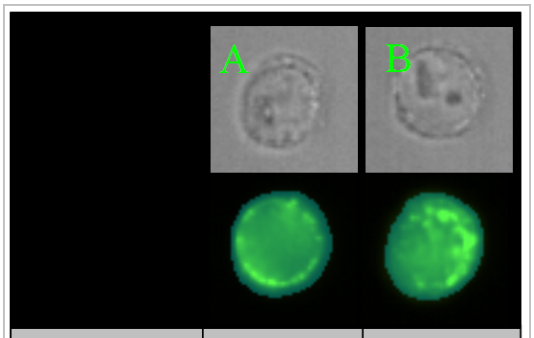
Application Examples:

- Estimate the average fluorescence activity. This feature is preferred over the Raw Mean Pixel feature.
- Quantify relative levels of mean fluorescence between cells.
- Identify bright punctate spots by calculating the max to mean pixel ratio.
- Track internalization of surface bound antibodies.

Median Pixel Feature

The Median Pixel feature is the median of the background-subtracted pixels contained in the input mask. It is more robust than the mean as an estimate of the average fluorescence since it is less influenced by outliers.

The relationship of Max, Mean, Median and Min Pixel is shown in the figure below:



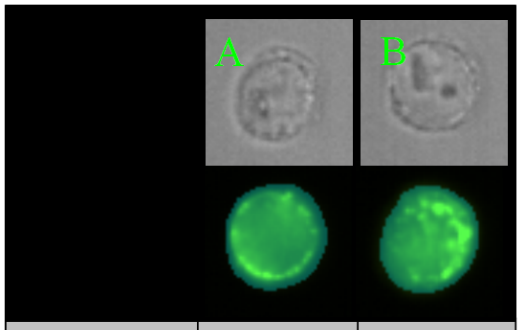
FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

Application Example:

– Estimate the average fluorescence activity. This feature is preferred over the Raw Median Pixel feature.

Min Pixel Feature

The Min Pixel feature is the smallest value of the background-subtracted pixels contained in the input mask. There will be some negative numbers due to the background subtraction, therefore the Raw Min Pixel feature is preferred.



FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

Application Examples:

- Obtain the minimum value in an image after background subtraction. Very likely to be negative in brightfield imagery.
- Quantify spectral absorbance using the brightfield image.
- Identify over compensated images.

Raw Intensity Feature

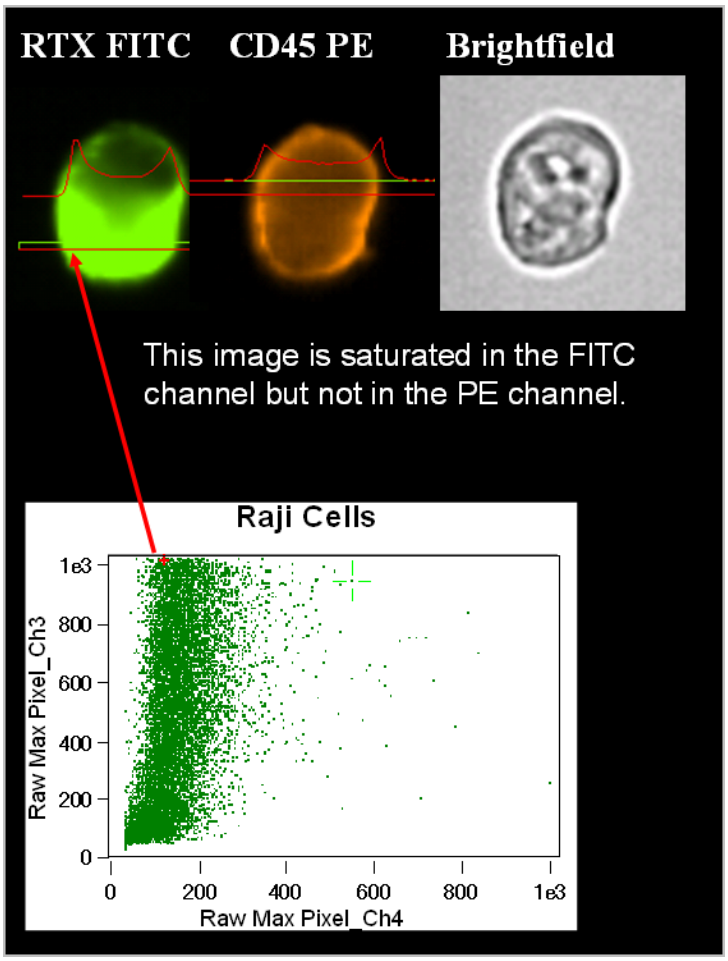
The Raw Intensity feature is the sum of the pixel values within the mask including camera background.

Application Example:

– Estimate raw fluorescence activity. This feature is less relevant than the Intensity feature because it includes camera background intensity.

Raw Max Pixel Feature

The Raw Max Pixel feature is the largest value of the pixels contained in the input mask.



Application Examples:

- Determine the presence of saturated events.
- May also be used to estimate the peak fluorescence activity, though the Max Pixel feature is recommended for this application.
- Measure the maximum pixel value within the mask.
- Identify cells that saturate the CCD, Saturation Count feature can also be used for this application.

Raw Mean Pixel Feature

The Raw Mean Pixel feature is the mean of the pixels contained in the input mask. This is computed as Raw Intensity/number of pixels.

Application Example:

– Estimate the raw average fluorescence activity. This feature is less relevant that the Mean Pixel feature.

Raw Median Pixel Feature

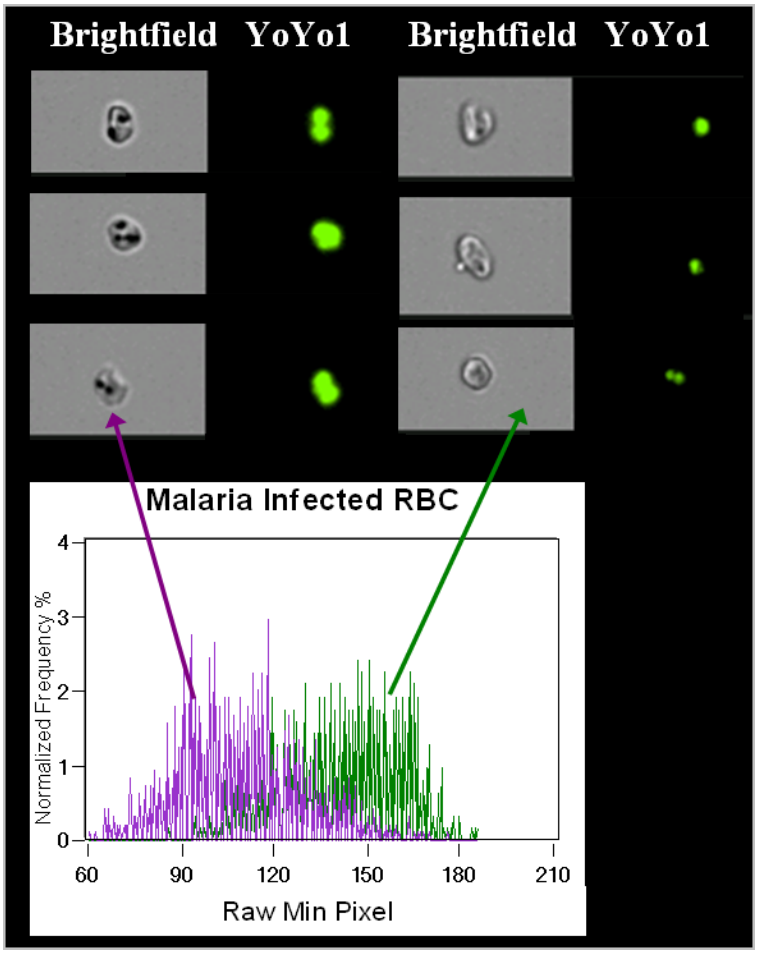
The Raw Median Pixel feature is the median of the pixels contained in the input mask.

Application Example:

- Estimate the raw average fluorescence activity that is robust to outliers. This feature is less relevant than the Median Pixel feature.

Raw Min Pixel Feature

The Raw Min Pixel feature is the smallest value of the pixels contained in the input mask. The example below illustrates quantifying the level of malarial infected cells by using Min Pixel values of brightfield imagery.



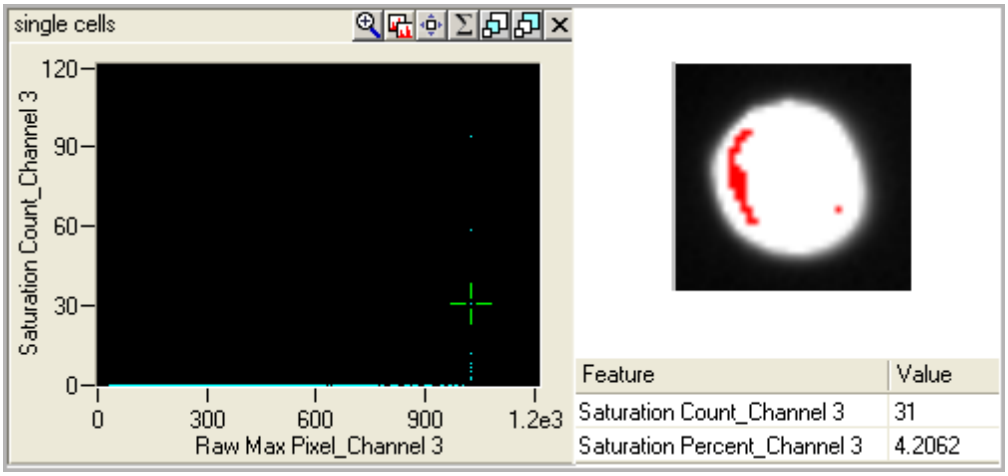
Application Example:

- Quantify spectral absorbance using the brightfield image.
- Identify over compensated images.
- Measure the level of malaria infection in RBCs.

Saturation Count Feature

The Saturation Count feature reports the number of saturated pixels in an object. See also Saturation Percent Features.

In the figure below, objects with saturated pixels are lined up at the Raw Max Pixel value of 1023 and a selected image is shown with saturated pixels in red.



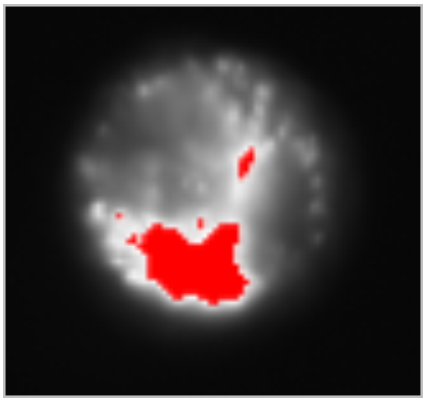
Application Example:

– Measure the validity of the experiment setup. Saturated data may not produce useful information.

Saturation Percent Features

The Saturation Percent feature reports the percentage of saturated pixels in an image. Pixel intensities are measured on the camera pixels from 0 to 1023 (10 bit) and therefore become saturated and cannot be quantified after 1023. See also Saturation Count Feature.

An object with saturated pixels shown in red:



Application Example:

- Measure the validity of the experiment setup. Saturated data may not produce useful information.

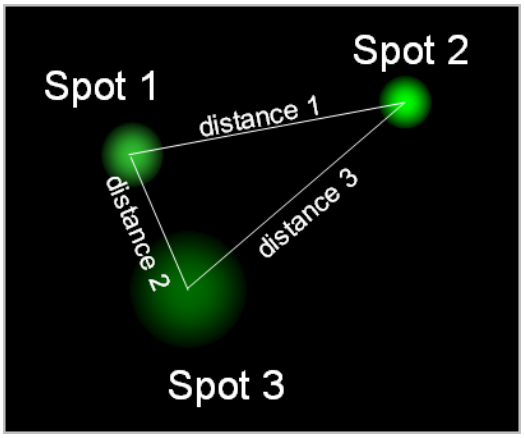
Spot Intensity Min and Spot Intensity Max Features

Spot Intensity Min provides the smallest Raw Mean Pixel value (not background subtracted) of the dimmest spot (connected component). The Raw Mean Pixel values for each spot is computed and the smallest value is reported.

Spot Intensity Max provides the largest Raw Mean Pixel value (not background subtracted) of the brightest spot (connected component). The Raw Mean Pixel values for each spot is computed and the largest value is reported.

These are two of four features that can be used to identify objects with spots that are close together, dim, bright or small when counting spots in an image. To use these features, the spots need to be individually masked, such as using the Spot or Peak Mask. The Spot Area, Distance, and Intensity Min or Max features measure properties of different spots in an image and are often used with the Spot Count feature (under Texture). Spot Area Min (Size) provides the area of the smallest spot. Spot Distance Min (Location) provides the shortest distance between two spots. See also Spot Area Min Feature, Spot Distance Min Feature, and Spot Count Feature.

The following diagram illustrates these features:



- Spot Area Min is the Area of spot 1
- Spot Distance Min is distance 2 in microns
- Spot Intensity Max is the Raw Mean Pixel value of spot 2
- Spot Intensity Min is the Raw Mean Pixel value of spot 3

Application Example:

– In FISH Spot Counting, these features are used to identify ambiguous spots that are located too close together, too dim, to bright or too small to count and can be eliminated from the analysis.

Uncompensated Intensity

The Uncompensated Intensity feature is the sum of the background subtracted pixel values within the masked area of the image with no compensation applied. This is the Intensity of the uncompensated image. This feature is calculated in INSPIRE during acquisition.

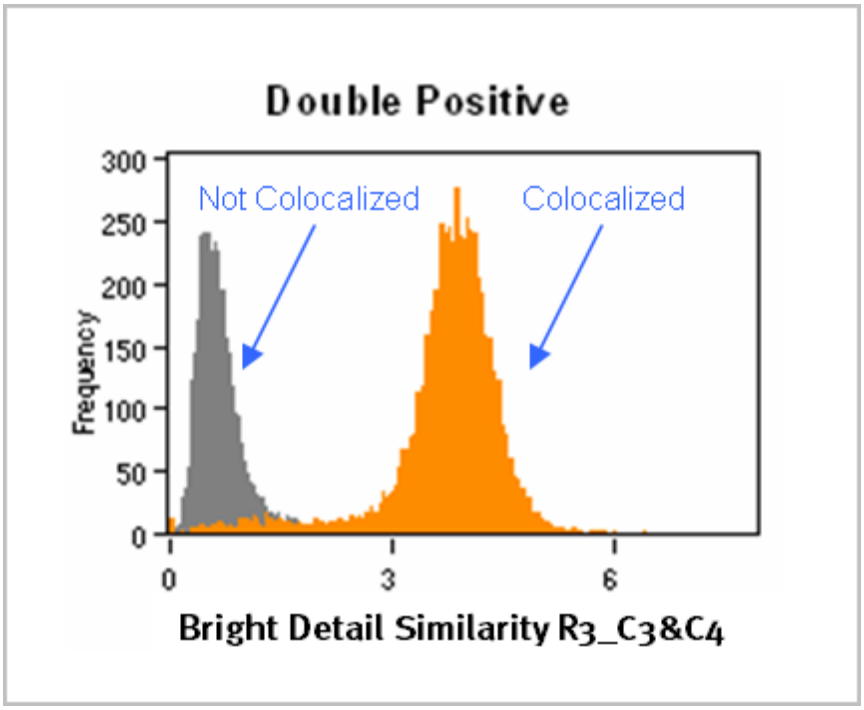
Understanding the Comparison Features

The Comparison features describe the difference of intensity measurements between masks or pixels in different images or the same image with different masks. These include Bright Detail Similarity R3, Intensity Concentration Ratio, Internalization, and Similarity.

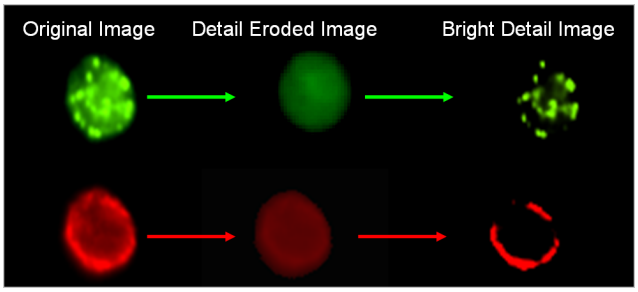
Bright Detail Similarity R3 Feature

The Bright Detail Similarity R3 feature is designed to specifically to compare the small bright image detail of two images and can be used to quantify the co-localization of two probes in a defined region, such as that of endosomes. The Bright Detail Similarity R3 feature is the log transformed Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the masked area in the two input images. Since the bright spots in the two images are either correlated (in the same spatial location) or uncorrelated (in different spatial locations), the correlation coefficient varies between 0 (uncorrelated) and 1 (perfect correlation), and does not assume negative values. The coefficient is log transformed to increase the dynamic range between {0, inf}.

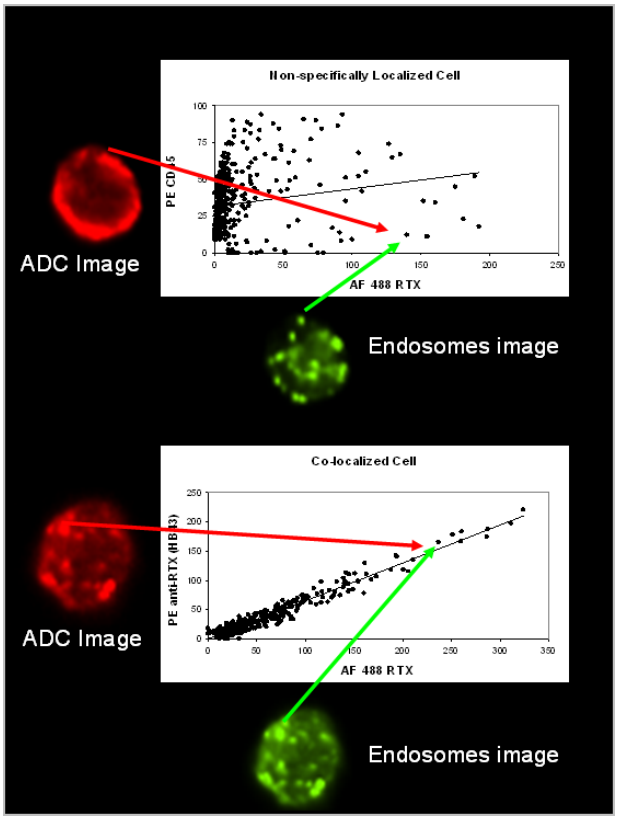
The following figure shows the Bright Detail Similarity R3 graph of two populations, one that has colocalization and one that has no colocalization.



The figure below illustrates the process of obtaining the localized bright spots. The bright areas are eroded from the original image and the detail eroded image is subtracted from the original image resulting in the bright detail image.



The figure below shows the correlation analysis between an image pair.



Application Examples:

- Quantify the degree of colocalization between two probes.
- Track internalization and intracellular trafficking of antibody drug conjugates to either the endosomes or the lysosomes.
- Colocalization of Rituxan and compliment C3b.
- Used in the Co-localization Wizard

Intensity Concentration Ratio Feature

The intensity concentration ratio is defined as the ratio of the intensity inside the first input mask to the intensity of the union of the two masks - the higher the score, the greater the concentration of intensity inside the first mask. All pixels are background-subtracted. The ratio is invariant to cell size and can accommodate concentrated bright regions and small dim spots. The ratio is mapped to a log scale to increase the dynamic range to values between {-inf, inf}. This feature is a generalization of the Internalization feature. See Internalization Feature for more information.

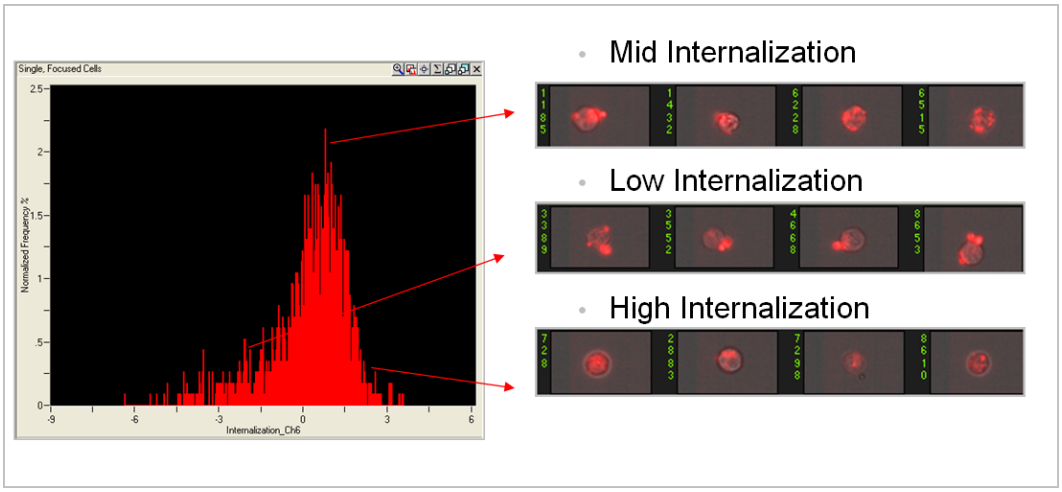
Application Example:

- Quantify relative intensity concentrations between different cellular compartments. Internalization is a special case of this where the first mask is the internal compartment and the second is the membrane region.

Internalization Feature

The Internalization feature is defined as the ratio of the intensity inside the cell to the intensity of the entire cell. The higher the score, the greater the concentration of intensity inside the cell. All pixels are background-subtracted. The user must create a mask to define the inside of the cell for this feature (see About Masks and Overview of the Mask Manager). The feature is invariant to cell size and can accommodate concentrated bright regions and small dim spots. The ratio is mapped to a log scale to increase the dynamic range to values between {-inf, inf}. Internalized cells typically have positive scores while cells with little internalization have negative scores. Cells with scores around 0 have a mix of internalization and membrane intensity.

Composite Images of brightfield and channel 6 are shown for High, Medium, and Low Internalization values.



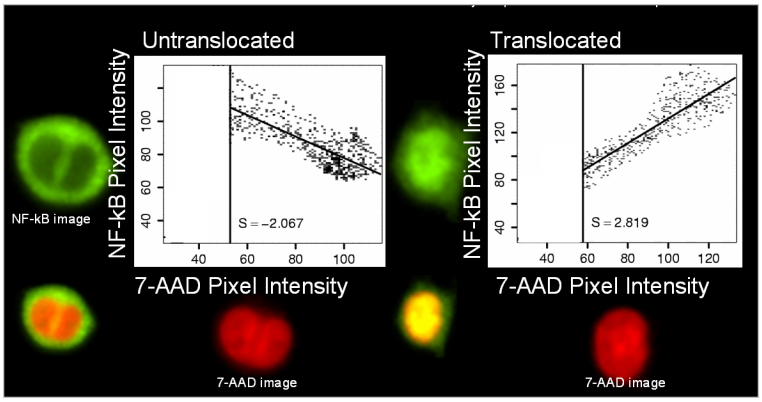
Application Examples:

- Quantify internalization when supplied with the internal mask.
- Quantify the intensity ratio of a region of interest to the whole cell.
- Used in the Internalization Wizard.

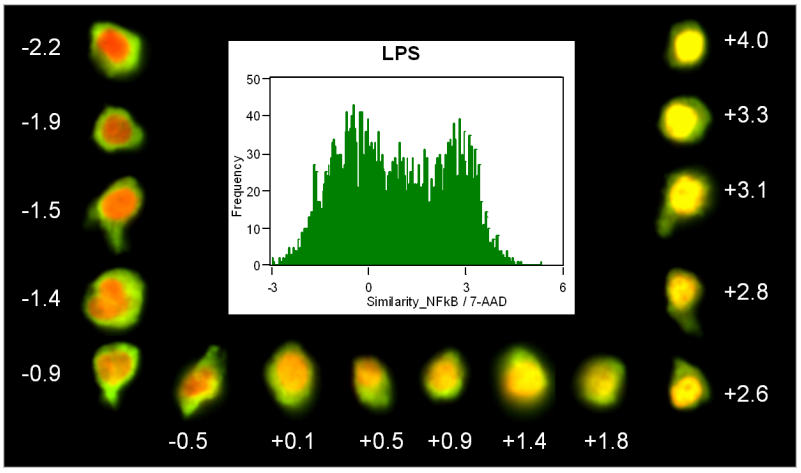
Similarity Feature

The Similarity feature is the log transformed Pearson's Correlation Coefficient and is a measure of the degree to which two images are linearly correlated within a masked region.

The following figure shows two image pairs that are in spatial registry to one another. On the left the NF-*k*B (green) is predominantly located in the cytoplasm of the cell and has a dissimilar distribution compared to the 7-AAD image (red). When the intensity of the green is high, the intensity of the red is low and vice versa. The Similarity value for this cell is -2.067 indicating that the image pair has a high degree of dissimilarity. Analysis of the image pair on the right shows that when the intensity of the green is high, the intensity of the red is high and the Similarity value is a high positive number.



Below are examples of cells with varying amounts of similarity between the NF*k*B image in green and 7-AAD image in red shown here as a composite image. The most dissimilar image pairs in the upper left to the most similar image pairs in the upper right.



Application Examples:

- Quantify translocation.
- Identify copolarization of two probes.
- Used in the Nuclear Localization Wizard

XCorr Feature

The XCorr feature is a measure of similarity or 'sameness' between two images - the higher the value, the more similar the images. It is robust to intensity variations and relative shifts between the images and is typically used with the combined mask MC. It is computed using the normalized cross correlation between the two input images.

Application Examples:

- Used as a mask-independent measure of similarity between two images.

Understanding the System Features

The system features do not require a mask.

Camera Line Number Feature

The Camera Line Number feature returns the camera line number values. This feature is obtained from INSPIRE.

Application Example:

- Used in objects per mL feature.

Camera Timer Feature

The Camera Timer feature returns the camera timer values that are in ticks. This feature is obtained from INSPIRE.

Application Example:

– Used in Time feature.

Flow Speed Feature

The Flow Speed is the calculated flow speed, in mm/sec, of the object.

The Flow Speed feature is the speed of flow of the cells. It is obtained from INSPIRE. It should be very consistent across all cells in a file.

Application Example:

- Determine consistency of flow.

Object Number Feature

The Object Number feature denotes the serial number of a cell in a file.

Application Example:

– Reference an object in a file.

Objects/ml Feature

The Objects per mL feature returns the object concentration with respect to local volume.

Application Example:

– Monitor the object flow during the run. Note: Use the statistic Concentration to obtain objects/ml of a population.

Objects/sec Feature

The Objects per sec feature returns the local object concentration with respect to time.

Application Example:

- Monitor the throughput during a run. Note: Use the statistic Concentration to obtain objects/ml of a population.

Time Feature

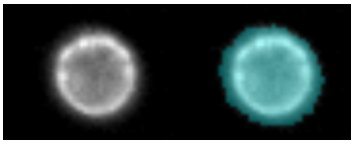
The Time feature returns the camera timer values that are in ticks, converted to secs with a formula.

Application Example:

- Obtain the time taken to collect a sample

About Masks

The set of pixels that contains the region of interest is called the mask. In the following picture, the mask consists of the set of pixels on the right image that are colored cyan. The cell is represented in the greyscale image on the left. Calculating some feature values, such as the Area value, requires only a mask. Calculating others, such as Intensity value, requires a mask and a channel image.

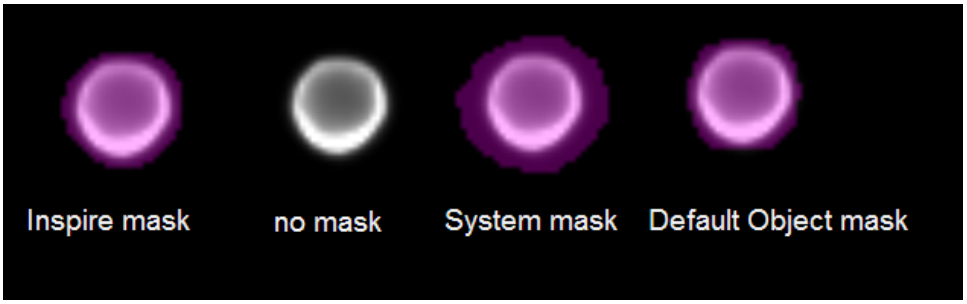


There are three types of masks: Default masks, Combined Masks and Function Masks:

1 Default masks named M01 through M12 are either created in INSPIRE during acquisition or created in IDEAS when a .rif file is opened. The default mask used by INSPIRE during acquisition (Inspire) is different than the default mask created in IDEAS (Default Object) when a .rif file is opened with QI or from an ImageStreamX. These masks are stored in the .cif file and cannot be changed by the user.

Conversion note: Versions of IDEAS prior to 3.0 were using the System function mask with a weight of 5 for the default masks which was more permissive and resulted in larger masks.

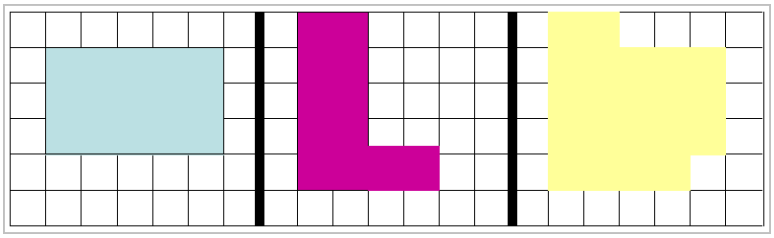
Below is an example of the difference between the default masks.



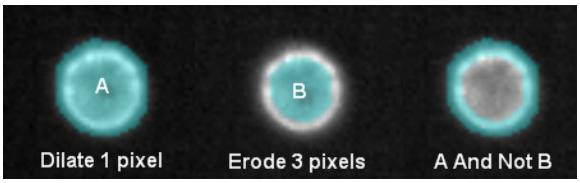
2 Combined masks are created using Boolean logic to combine and subtract masks. For example, the cytoplasmic mask is created by taking the brightfield mask and not the morphology mask of the nuclear image.

You can use the Mask Manager to combine masks of different regions or images. The IDEAS application default template provides a combined mask named MC that is the union of the pixels from all channel masks and a NMC mask that is everything outside of MC. The following illustration shows two channel masks

that are combined into one mask, which is shown in the right-most panel.



Below is an example of making a membrane mask using Boolean Logic.

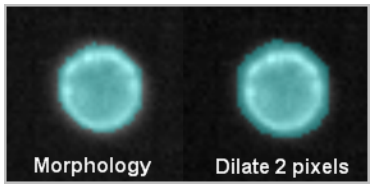


3 Function masks are created with user input. There are fourteen types of function masks, Dilate; Erode; Fill; Inspire; Intensity; Interface; Morphology; Threshold; Spot; System; Object; Peak; Range; Skeleton; and Valley. Each of the functions masks are defined here.

Refer to Creating New Masks with the Mask Manager for more details about how to create new masks.

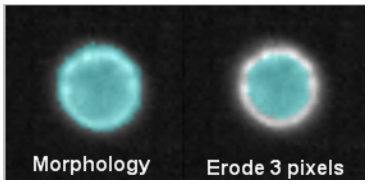
Dilate Mask

The Dilate mask adds the selected number of pixels to all edges of the starting mask.



Erode Mask

The Erode mask removes the selected number of pixels from all edges of the starting mask.



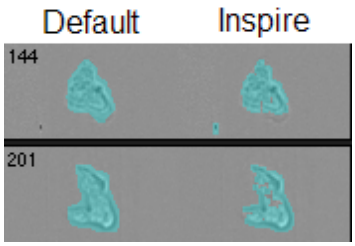
Fill Mask

The Fill mask fills in any holes in the starting mask.

Inspire Mask

The Inspire mask masks pixels above background and is the mask used during data acquisition in INSPIRE. This mask is available to understand what is being masked during collection and is not generally used for feature calculations.

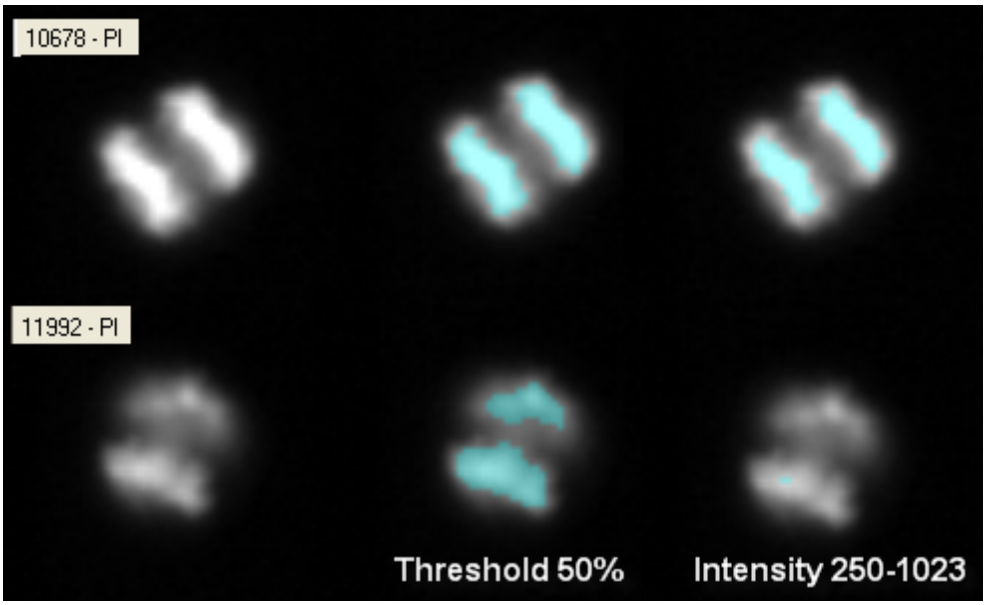
Note: this mask is new in IDEAS versions 4.0 or later.



Intensity Mask

The Intensity mask masks pixels between the lower and upper raw intensity thresholds not background subtracted. See also Threshold Mask.

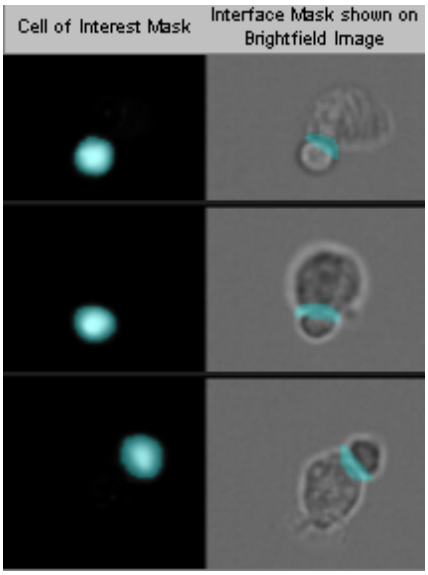
In the example below, cell #10678 is bright and cell #11992 is dim. The 50% Threshold mask is similar for both images whereas the Intensity mask 250+ is quite different, since only a few pixels in the dim image are greater than 250 counts, while most of the metaphase plates in the bright image are masked.

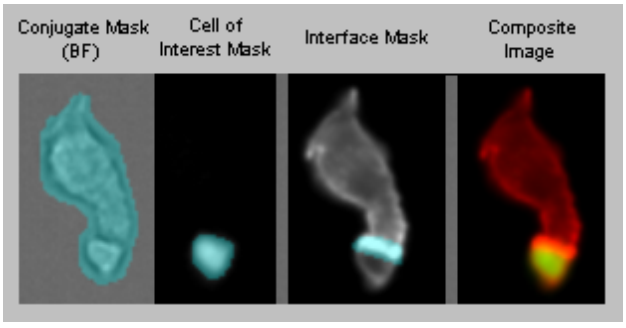


Interface Mask

The interface mask identifies pixels in an object where the object is in contact with a second object. Three input parameters are defined. First, the mask of one of the objects (cell of interest). Next, the mask that covers both objects (conjugate). A close fitting mask using another function mask such as Object (tight) can be used for the cell of interest mask. A brightfield mask can be used for the conjugate. Finally, the width of the interface mask from the contact point towards the cell of interest is entered.

Examples are shown below:





Application Examples:

– Used to quantify synapses in T cell APC (antigen presenting cell) conjugates.

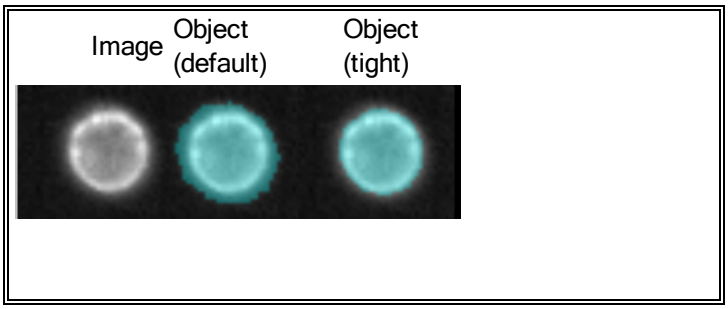
Morphology Mask

The Morphology mask includes all pixels within the outermost image contour. This mask, which is used in fluorescence images, is best used for calculating the values of overall shape-based features.

Object Mask

The Object mask segments images to closely identify the area corresponding to the cell. It is based on the assumption that background pixels exhibit high uniformity to each other. This helps distinguish the background from the cell pixels. The mask characterizes the background pixels using a set of features and then segments the image by determining all the pixels that deviate from the background feature set. The default option is used for the default segmentation masks. The tight option uses a different set of features to characterize the background which results in a tighter fit around the cell.

Examples are shown below:



Application Examples:

- Used to get a close fit around the cellular area (tight option).
- Can be used in lieu of the morph mask for applications where the morph is so tight that it provides incomplete masking, sometimes splitting cells into two regions, such as a nuclear dye image of cells in anaphase or telophase.
- Can be used in lieu of the morphology mask with the Similarity feature when measuring nuclear translocation for better separation between untranslocated and translocated cells (tight option).
- Used as the default segmentation masks (default option).

Peak Mask

The Peak mask identifies intensity areas from an image that have local maxima (bright) or minima (dark). Initially, the peak mask will identify all peaks in the image. To select peaks which have certain brightness, the spot to cell background ratio is used. This is the ratio between the spot pixel value to the mean camera background value in the original image.

Below is an example of the Peak, bright option.

These peak areas
will be masked

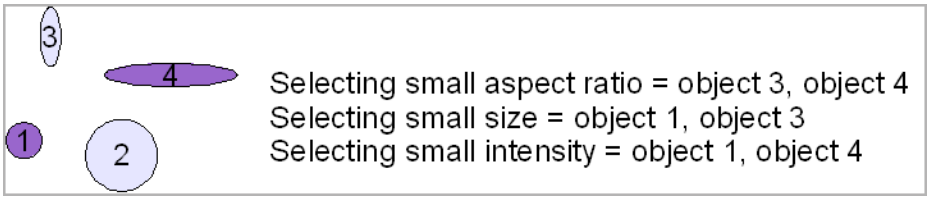
Application Examples:

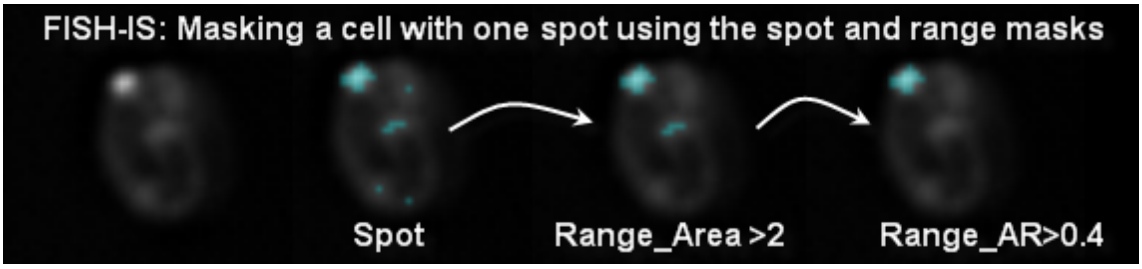
- Used with the Spot Count feature to quantify the speckleness of cells.
- Separate connected spots in a Spot Mask into individual components.

Range Mask

The Range mask provides a capability to select components in an image within a selected size and/or aspect ratio by setting a minimum and maximum area and minimum and maximum aspect ratio.

To select pixels within a range of intensity values, see Intensity Mask.


3
4
1
2
Selecting small aspect ratio = object 3, object 4
Selecting small size = object 1, object 3
Selecting small intensity = object 1, object 4

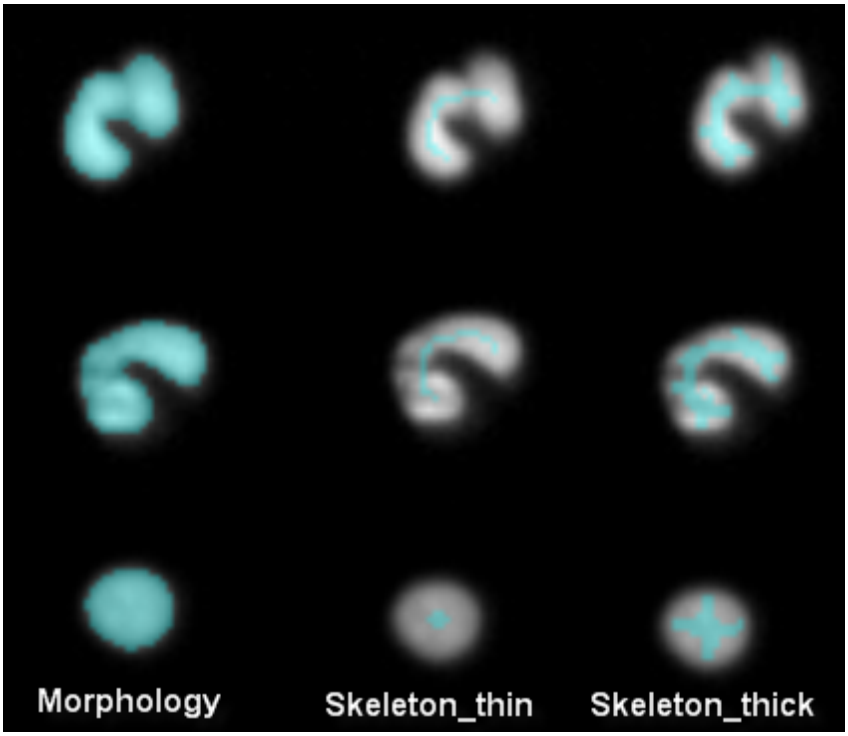


Application Examples:

- Use with a Spot Mask to constrain the Spot Count feature to round spots.
- Use on any other mask that has multiple components to define unwanted objects such as debris, objects that are too small or whose shapes are not circular.

Skeleton Mask

The skeleton mask provides the barebone structure of the object from the starting mask. Two options are available: thin or thick skeletons. The thin option produces the condensed shape of the object and typically takes a form of 1-pixel wide skeletal line. The thick option is intensity weighted. The thin option is dependent on the shape of starting mask; thick uses the pixel intensities and is less sensitive to the shape of the starting mask. The user will need to pay careful attention to the starting mask. In the example below the Morphology mask of the image was used as the starting mask for creating the skeleton.



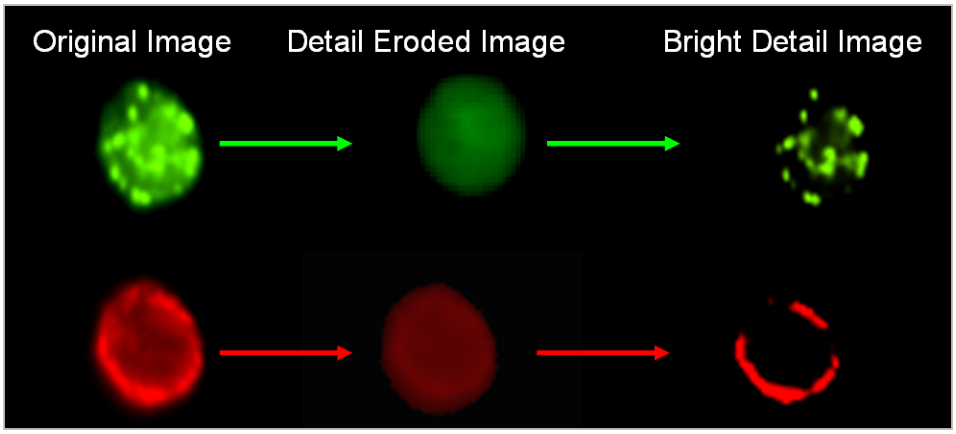
Application Examples:

- Thick skeletons can be used with shape-based features such as symmetry to accentuate the shape of an object, and provide greater separations.
- Separate singlets and doublets by computing the area of the thin skeleton mask. We have used the object(tight) for this case.
- Nuclear morphology measurements with lobe count feature for cell classification cells.

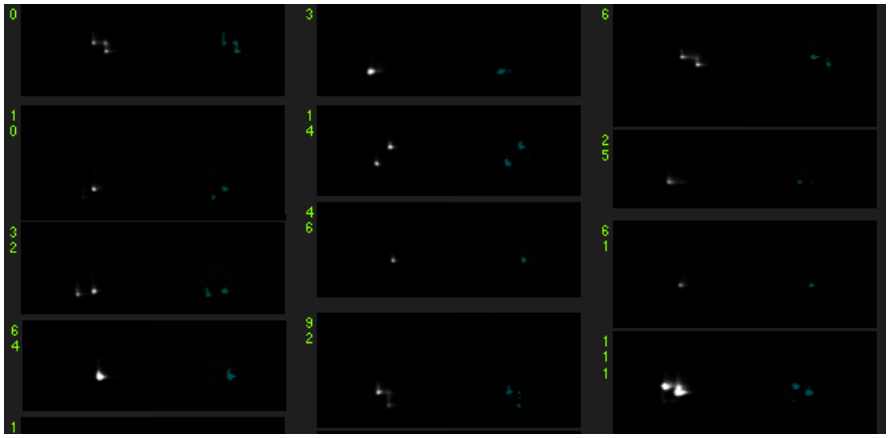
Spot Mask

The Spot Mask has two options: bright or dark. The bright option obtains bright regions from an image regardless of the intensity differences from one spot to another. The ability to extract bright objects is achieved using the an image processing step that erodes the image and leaves only the bright areas. The dark option obtains dark regions. The spot to cell background ratio and radius are specified by the user. The spot to cell background ratio is the spot pixel value divided by the background in the bright detail image. A radius value of *x* implies that the image contains spots with thickness of *2x+1* pixels.

The figure below illustrates the open residue process. The bright areas are eroded from the original image and the detail eroded image is subtracted from the original image resulting in the bright detail image.



The image pairs below show objects in grayscale next to their corresponding Spot Masks in cyan. Spot masks can be further refined using the Peak and/or Range masks. See Peak MaskRange Mask.



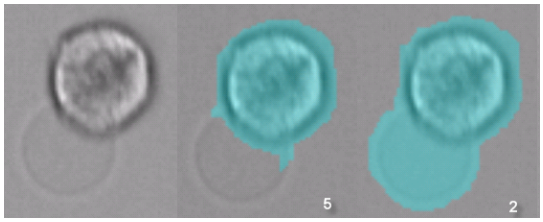
Application Examples:

- Used with the Spot Count feature to enumerate spots in images such as for FISHIS®.
- Used with Intensity features to quantify intensity in spots.
- Dark spot finds valleys in images such as the low intensity between 2 stained nuclei and is useful for finding immune synapses.
- Identifies the dark areas in red blood cells or parasitic infections in bright-field imagery.

System Mask

The System mask segments objects in an image based on a probability model of how pixels should be grouped together. The user sets a weight value that defines a loose or tight grouping. A low weight value groups in a more permissive manner.

Shown is an example of a cell with a apoptotic bleb that is not masked with the System mask weight set at 5 but is masked with the System mask weight set at 2.



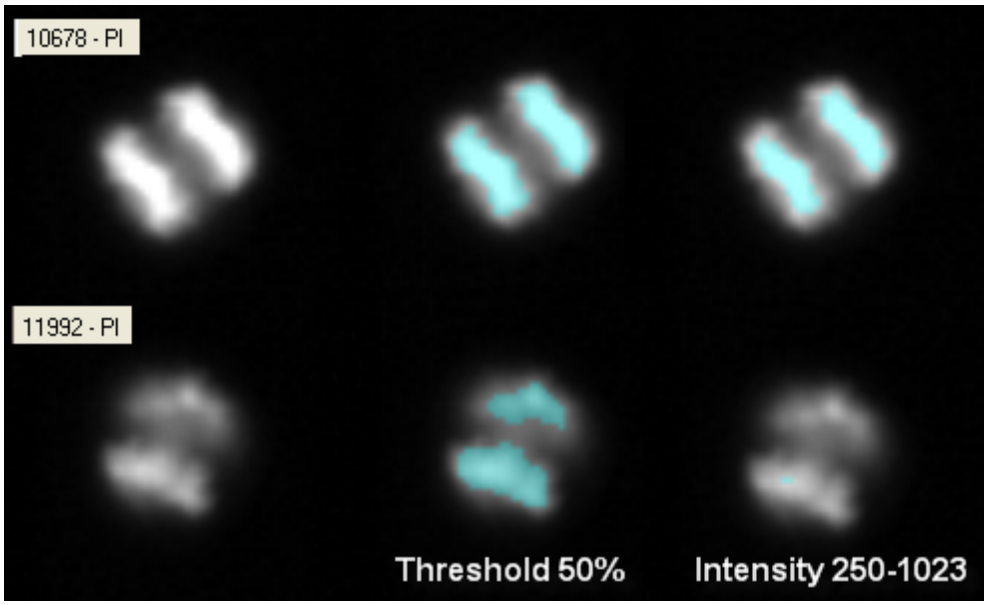
Application Example:

- Used on brightfield images to capture a low contrast areas such as cells that undergo a blebbing process, tails of sperm or other low contrast type of structures.

Threshold Mask

The Threshold mask is used to exclude pixels, based on a percentage of the range of intensity values as defined by the starting mask. The user chooses the starting mask when creating the Threshold mask. See also Intensity Mask.

In the example below, cell #10678 is bright and cell #11992 is dim. The 50% Threshold mask is similar for both images whereas the Intensity mask 250+ is quite different, since only a few pixels in the dim image are greater than 250 counts, while most of the metaphase plates in the bright image are masked.

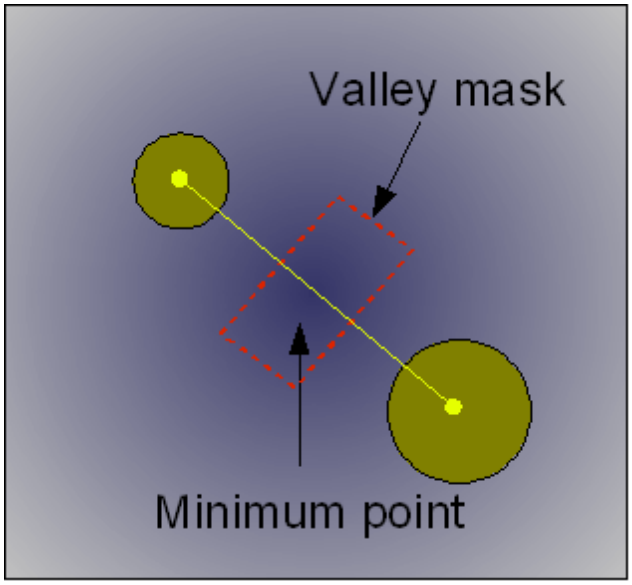


Application Example:

- Used with the Area feature to define apoptotic cells in the Apoptosis Wizard.

Valley Mask

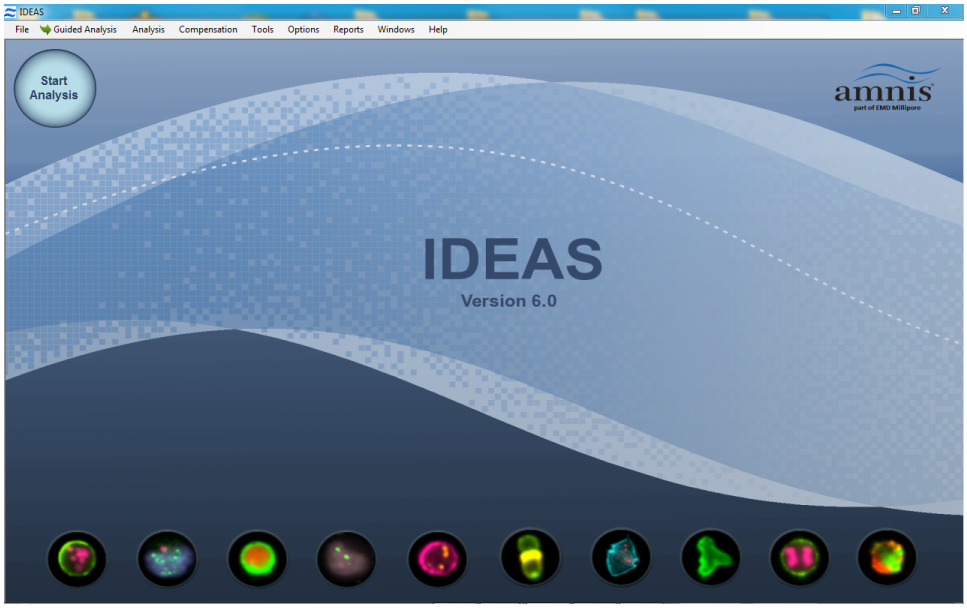
The Valley mask is a rectangular mask that sits between two bright regions in a starting mask, such as between two nuclei. It is constructed by finding the minimum intensity along the skeletal line between these two bright regions. The skeletal line is obtained internally using the skeleton (thin) masking as described in Skeleton Mask. This minimum intensity identifies the intersection between the two objects. The mask is drawn perpendicular to this skeletal like. The length of the valley mask rectangle is equal to the minor axis of the object and the width of the mask is defined by the user in pixels.



Application example:

– Quantify the intensity of a probe in an immune synapse.

Appendix: Troubleshooting



Troubleshooting

This chapter covers common issues and provides solutions.

Always make sure you are using the most up-to-date version of IDEAS by logging in to your account at www.amnis.com and downloading the software.

Application Hanging

If the IDEAS application is hanging, there may be a memory issues, especially with large file processing. You must use the Task Manager to force quit the application.

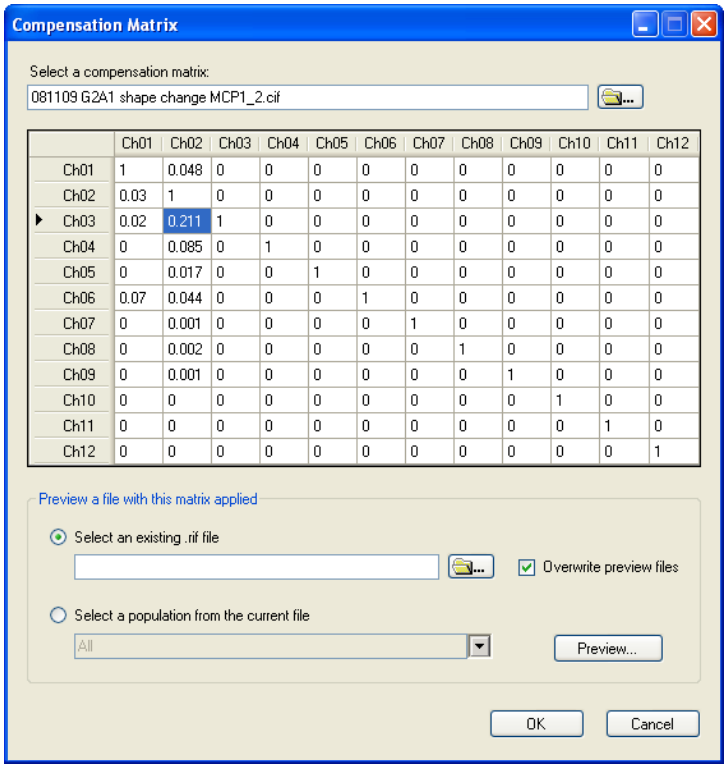
1 Press and hold Ctrl + Alt + Delete.
2 The Window Task Manager appears.
3 Under the **Applications** tab, select IDEAS Application.
4 If the status is Not Responding, select **End Task**.
5 The manager will force quit the application after a confirmation.

Compensation

Sometimes an applied matrix produces poorly compensated data. This can happen for a number of reasons: 1) miscalculation of the compensation matrix by inclusion of inappropriate events (such as doublets, saturated pixel events, or artifacts), 2) controls used for matrix calculation differ significantly from the experimental samples (different cell type, different probe), or 3) cells exhibit substantial autofluorescence. This protocol describes a method for manually adjusting and validating a compensation matrix for difficult samples.

To troubleshoot and repair a compensation matrix:

1 Create a population of cells that are miscompensated using the tagging tool. See Creating Tagged Populations. Choose single cells that are exhibiting crosstalk. Choose a range of intensities from negative to bright but not saturated, preferably single color. If single color cells are not available, choose cells with a distinct staining pattern in the peak channel.
2 Create Intensity scatter plots of adjacent channels in order to observe the over- or under-compensation.
3 Identify the matrix values that need adjusting by inspecting the scatter plots and images. Each column contains the coefficients for the peak channel into the corresponding crosstalk channels (rows). For example the crosstalk of channel 2 (green) into channel 3 is highlighted in the matrix below.



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.048	0	0	0	0	0	0	0	0	0	0
Ch02	0.03	1	0	0	0	0	0	0	0	0	0	0
Ch03	0.02	0.211	1	0	0	0	0	0	0	0	0	0
Ch04	0	0.085	0	1	0	0	0	0	0	0	0	0
Ch05	0	0.017	0	0	1	0	0	0	0	0	0	0
Ch06	0.07	0.044	0	0	0	1	0	0	0	0	0	0
Ch07	0	0.001	0	0	0	0	1	0	0	0	0	0
Ch08	0	0.002	0	0	0	0	0	1	0	0	0	0
Ch09	0	0.001	0	0	0	0	0	0	1	0	0	0
Ch10	0	0	0	0	0	0	0	0	0	1	0	0
Ch11	0	0	0	0	0	0	0	0	0	0	1	0
Ch12	0	0	0	0	0	0	0	0	0	0	0	1

- Undercompensation (crosstalk coefficient is too low):

  Plots: Intensity mean for the single color positive population is higher than the unlabeled population in the crosstalk channel or the intensity in the crosstalk channel trends diagonally upwards.

  Images: the crosstalk channel contains an apparent fluorescent mirror-image.
- Overcompensation (crosstalk coefficient is too high):

  Plots: Intensity mean for the single color positive population is lower than the unlabeled population in the crosstalk channel or the intensity in the crosstalk channel trends diagonally downwards.

  Images: the crosstalk channel contains dark spots corresponding to the bright spots in the fluorescent channel of interest.

4 In the Compensation menu choose **View/Edit Matrix** and manually change the incorrect crosstalk matrix values identified above. Start with changes of ~.1 or ~.05 and use smaller and smaller increments as you refine the matrix.

5 Click **Preview** and choose the tagged population to view the results of the changed coefficient.

6 Repeat steps 4 and 5 until the matrix is corrected.

7 Click **Save**, append manual to the matrix name, then click OK.

8 Open the .cif file and use the new matrix to create a new .daf file.

Creating a TIFF

If you cannot see the TIFF image that you created, trying changing the resolution to 8-bit.

Deleting a Population and Region

Often, a user deletes a population but forgets to delete the region. Deleting a population does not delete the region. You must delete the region itself.

Object Number set to Zero

When opening a .daf file, there may be an error if the object number is set to zero. This can happen if the data was collected during a crash within INSPIRE. This error can be corrected with the following procedure.

1 Select **Tools** > **Merge .rif Files**.
2 Click **Add Files** to select the single .rif file.
3 Click **OK**. Enter a new name if desired. The single .rif file will merge with itself and rewrite the file with the proper object count.

Buttons or options in windows are not appearing

When the font size setting is set to large some windows will not size properly causing buttons or text boxes to not appear. To change the font size in Windows go to the Control Panel>Display>Appearance and select Font size Normal.

Glossary

Table 1: Glossary of Terms

Term	Definition
acquisition	The process of collecting data from the ImageStream cell analysis system.
brightfield	A type of illumination that uses transmitted light. On the ImageStream cell analysis system, this light is provided by a halogen lamp.
brightfield image	An image that is produced by transmitted light. On the ImageStream cell analysis system, this light is provided by a halogen lamp.
brightfield	The camera channel that the brightfield image appears in.
calibration	The precise adjustment of instrument components based on test results for the purpose of optimizing functionality.
CCD	See charge-coupled detector (CCD).
	One of the six physical partitions on the camera. Each camera channel collects a different spectral band of imagery, which allows for the collection of brightfield, darkfield, and up to four fluorescence images per object.
charge-coupled detector (CCD)	A sensor for recording images that consists of a particular type of integrated circuit—one that contains an array of linked, or coupled, capacitors. Under the control of an external circuit, each capacitor can transfer its electric charge to either of its neighbors.
coefficient of variation (CV)	The mean-normalized standard deviation, expressed as a percentage. The CV measures the variation of a feature value independent of the population mean value. The formula is: CV = 100 × standard deviation / mean
CV	See coefficient of variation (CV).
compensation	The process of removing intensity—specifically, intensity that was derived from fluorescence crosstalk that originated from dyes centered in other channels. The IDEAS application performs compensation on a pixel-by-pixel basis.
compensation matrix	The set of values that report the relative amount of fluorescence of each probe in each channel. The compensation matrix is used to subtract intensity originating from dyes centered in other channels.
crosstalk	Leakage of fluorescence signal from a fluorochrome into adjacent channels.
darkfield	A type of illumination in which the sample is illuminated at angles that do not directly enter the objective. On the ImageStream cell analysis system, 90-degree angle side scatter from the 488-nm laser provides the darkfield imagery.
FISH	See fluorescent in situ hybridization (FISH).
fluorochrome	A fluorescent dye used to label cellular constituents or specific probes of cellular constituents.
fluorescence	Light emitted by a fluorescent dye following excitation.
fluorescence com-	The adjustments made to remove the fluorescence emissions of a

Term	Definition
pensation	fluorochrome into adjacent channels.
fluorescent in situ hybridization (FISH)	A physical mapping approach that uses fluorescent tags to detect the hybridization of probes with metaphase chromosomes or the less-condensed somatic interphase chromatin.
gain	The amplification of a detector signal.
grayscale	The brightness level, ranging from black to white, of a pixel or group of pixels.
pixel	A pixel is equal to a half micron in length with the 40X objective, 1 micron with the 20X objective and 0.33 microns with the 60X objective. Note that 1 pixel = x μm^2.
saturation	The state of a pixel that has a value at or above 1023 for the IS100 or 4095 for the ImageStream^X.
segmentation	The process of discriminating an object from its background.
spectral decomposition element	A custom set of longpass dichroic filters arranged in an angular array. The spectral decomposition element directs different spectral bands to laterally distinct channels on the detector. With this technique, an image is optically decomposed into a set of six sub-images, each corresponding to a different color component and spatially isolated from the remaining sub-images.
spatial offset	The registration error of the six channel images for a single cell. The spatial offset is measured during calibration and the values are saved to the image database.
Table of Coefficients	The table used by the compensation matrix to place the detected light that is displayed in each image into the proper channels, on a pixel-by-pixel basis.
template	A file that saves the set of instructions for an analysis session. Note that a template contains no data; it simply contains the structure for the analysis. This structure includes definitions of features, graphs, regions, and populations; image viewing settings; channel names; and statistics settings.

Index

A

Acquisition information 72

Advanced Analysis 49

Analysis Area

adding an image panel 103

adding text 110

overview 94

printing 145, 155

tools 94

Apoptosis

wizard 33

Application defaults 5, 132

Area 171

ast

about 19

B

Batch processing 74

Brightfield information 73

Building Blocks 47

Fluorescence Positives-one color 47

Fluorescence Positives-two color 47

Focus 47

Single Cell 47

Single Cell Default 47

Size/SSC 47

Tool 95

C

Camera settings information 73

Cell Classifiers 73

Cell Cycle

using a wizard 35

Channels collected 73

cif

merging 70

opening 53

saving 56

cif;about 18

Co-localization

using a wizard 37

Color

mapping dark/light mode defaults 10, 137

Show in Image Gallery 80

Compare, FlowSight, FlowSight QI, ImageStream 15

Compensation

adjusting compensation population 64

creating 59

definition 16

editing the matrix 67

overview 58

validating matrix 63

view matrix 73
Compensation matrix file
about 19
creating 59
Composites 85, 142
Copying images 87
Corrections information 72
ctm
about 19
creating 59

D

daf
about 18
opening 55
saving 56
Data analysis steps 22
Data analysis tools
about 78
Data files
ast 19-20
cif 18, 20
ctm 19-20
daf 18, 20
new from populations 71
opening 50
rif 18, 20

type 20
Defaults
application 5
color mapping dark/light mode 10, 137
compensation matrix 15
directories 6, 133
graphs 9, 135
image export 9, 136
mask 15
mask color 8, 134
population color 7, 134
population symbol 7, 134
statistics for a graph 8, 134
Diameter 172
Directories
changing default 6, 133
Display
making composites 81, 138
setting properties 81, 138
setting properties using a wizard 27
views in the Image Gallery 81, 138

E

EDF
kernels 73
Example data files 5

Exporting data 152
features 152
pixel intensity values 153

F

Feature Finder
wizard 29
Feature Manager
overview 113
tasks 117
tools 114
Features
angle 183
angle intensity 183
Area 171
aspect ratio 196
aspect ratio intensity 197
Bkdg mean 217
Bkgd std dev 218
bright detail intensity 206
camera line number 243
camera timer 244
categories 161
centroid delta x and y 185
centroid delta xy 187
centroid x and y 184
circularity 198

compactness 200

contrast 208

create combined 117

create multiple 115

create new 114

delete 118

diameter 172

elongatedness 201

Feature Finder Wizard 30

find a feature 118

flow speed 245

gradient max 210

gradient RMS 211

height 173, 181

intensity 219

internalization 238

lobe count 202

major and minor axis intensity 176

major axis and minor axis 175

max contour position 190

min pixel 224

modulation 213

object number 246

object per mL 247

object per sec 248

overview 159

perimeter 177

raw intensity 225
raw max pixel 226
raw mean pixel 227
raw median pixel 228
raw min pixel 229
saturation count 230
saturation percent 231
shape ratio 203
similarity 239
similarity texture R3 235
size 170
spot area min 178
spot count 214
spot distance min 192
spot intensity min 232
std dev 215
symmetry 2 204
table alphabetical list 162
table by category 164
thickness max 179
time 249
valley x and y 193
viewing 114
width and height 173, 181
without QI 169
File name extensions 5

Fluorochromes
table for IS100 61
table for ISX - 12 channel system 61
table for ISX - 6 channel system 61
Focus building block 47

G

graphs
copy and paste 102
Graphs
apply or remove region 102
creating 95
creating regions 99
defaults 9, 135
legend 98
moving 99
printing 156
resizing regions 101
statistics 97
zoom 101
Guided Analysis 23

H

Hardware requirements 4
Histogram tool 95

I

IDEAS
getting started 22
Image
copy 87
exporting defaults 9, 136
individual image 86
Image Display intensity mapping 83, 139
Image Gallery
channel view 80
composites 85, 142
overview 79
population 80
printing 145, 155
properties 81-82, 138
properties tool 79
resize 81
show-hide color 81
show-hide masks 80
tools 79
using 79
views 84, 141
Image panel size
change in Image Gallery 82, 139
Individual image
display properties 106

manipulating 86
measurement tool 104
pixel intensities 104
show-hide mask 108
Internalization
using a wizard 39

L

Laser information 73
Line Region Tool 95

M

Mask Manager
overview 88
tools 91
Masks
about 250
combining 91
creating new 88
default color 8, 134
dilate 252
edit 91
erode 253
examples 92
fill 254
intensity 256
list of 89
morphology 258

object 259
peak 260
range 261
show in Image Gallery 79
skeleton 262
spot 263
system 265
threshold 266
valley 267
viewing definitions 91
Merging cif files 70
Merging raw images files 69
Mitosis
using a wizard 35

N

Name and Color
Image Gallery 82, 139
Nuclear Localization
using a wizard 41
Nuclear translocation
using a wizard 41

O

Object Data 108
Object Feature Values Table 95
One color histogram building block 47

Opening files
using a wizard 26
Oval Region Tool 95

P

Pointer tool 94
Polygon Region Tool 95
Population Manager 124
tools 125
Population Statistics 111
Population Statistics table 95
Populations
creating 127
creating a new data file from 71
creating combined 125
deleting 124
display properties 124
during acquisition 73
viewing 124
Printing 145

R

Rectangle Region Tool 95
Region Manager 129
Regions
editing 129
viewing 129

Reporting 131
changing color mapping 144
graphs and statistics 146
Images 145
Images and Graphs 144
light mode graphs 144
statistics 148
statistics from multiple files 150
Reports
printing data 145, 155
rif
about 18
merge 69
opening 50

S

Sample Information
view 72
Saturation
view in Image Gallery 80
Saving data files 56
ast 57
cif 56
daf 56
Template 57
Scatter plot tool 95

Scatter plots
show-hide populations 102
Screen resolution 5
Select All Panels Tool 95
Shape Change
using a wizard 43
Single Cell Building Block 47
default 47
Size/SSC Building Block
default 47
Software requirements 4
Spot Count
using a wizard 45
Statistics
defaults for a graph 8, 134

T

Tagging tool 79, 95
Template file
about 19
default 15
definition 16
saving 57
Text tool 95
TIFs
creating 154
Two color scatter plot building block 47

V

Views

custom 84, 141

Volume information 72

W

Wizards

Apoptosis 33

Begin Analysis 28

Cell Cycle - Mitosis 35

Co-localization 37

Display Propertes 27

Feature Finder 29

Internalization 39

list 24

Nuclear Localization 41

Open File 26

Shape Change 43

Spot Count 45

Tool 95

Workflow; data analysis workflow 16

Z

Zoom

Image Gallery 80